

# Chapter Number

## Biosynthesis, Purification and Biotechnological Use of Exopolysaccharides Produced by Lactic Acid Bacteria

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### 1. Introduction

Polysaccharides have been used traditionally by the food industry for their viscosifying, emulsifying and biothickening properties and more recently for manufacture of functional food due to their prebiotic and immunomodulating properties.

Bacteria can synthesize cytoplasmic storage polysaccharides (e.g. glycogen), cell wall structural polysaccharides such as peptidoglycan, and lipoteichoic acids of gram-positive bacteria, and the lipopolysaccharides anchored in the outer membrane of gram-negative bacteria. In addition, some bacteria can secrete polysaccharide layers on their surface, which together with a few glycoproteins, constitute the glycocalyx. These exocellular polymers comprise the capsular polysaccharides, which form a cohesive layer or capsule covalently linked to the cell surface, and the exopolysaccharides (EPS), which form a slime layer loosely attached to the cell surface or secreted into the environment (Brock, 2008). The physiological role of these molecules are not yet clearly understood, although it is generally recognized that exocellular polysaccharides are not normally used as energy and carbon sources by the producing microorganism. They can serve for a variety of functions including cell recognition and interaction, adherence to surfaces and biofilm formation.

The majority of the polysaccharides used as additives by the food industry such as pectin, cellulose and alginate are obtained from plants and algae. However, other biopolymers like xanthan and gellan, also used as bio-thickeners, are synthesized by gram-negative bacteria. Furthermore, lactic acid bacteria (LAB) producing EPS are used mainly in the dairy industry for improvement of the rheological properties of fermented products as well as for the manufacture of functional food.

The taste/texture benefits of the EPS produced by LAB in fermented foods are well established, because these organisms produce polymers that improve the rheological

properties of dairy product. When they are added to food, polysaccharides show functions as thickeners, stabilizers, emulsifiers, gelling agents, and water binding agents (Kimmel et al., 1998). They also contribute to preservation, and enhance the organoleptic characteristics of milk and dairy products such as flavour and aroma (Macedo et al., 2002). More recently, these bio-molecules have been regarded as health promoters due to their role as prebiotics and/or the immunomodulatory properties linked to their structure. As a result, a number of studies are in progress in order to characterize the unmapped diversity of the EPS produced by LAB, since they are considered food-grade organisms.

In this chapter, we shall review the current knowledge pertaining to the EPS synthesized by LAB, from biogenesis to application, detailing their nature and structure. Moreover, the methods most frequently used for the production and purification of these biopolymers will be presented.

## 2. Composition, structure and classification of EPS

The EPS synthesized by LAB vary greatly in their chemical composition, structure and molecular weight. According to their chemical composition, EPS are classified into heteropolysaccharides (HePS) and homopolysaccharides (HoPS).

HePS are constructed of a backbone of repeated subunits that are linear or branched, with variable molecular masses (up to  $10^6$  Da). Each one of these subunits can contain between three and eight different monosaccharides and frequently has a range of different linkage patterns. The monosaccharides are present as the  $\alpha$ - or  $\beta$ -anomer in the pyranose or furanose form and D-glucose, D-galactose and L-rhamnose are the most frequently encountered. In few cases, N-acetylglucosamine, N-acetylglucosamine, manose, fucose, glucuronic acid and non-carbohydrate substituents (phosphate, acetyl and glycerol) are also present (de Vuyst & Degeest, 1999; de Vuyst et al., 2001).

Different strains of LAB isolated from dairy products, cereals and alcoholic beverages synthesize HePS. These belong to the genera *Lactococcus* (*L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis*), *Lactobacillus* (*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. casei*, *Lb. sakei*, *Lb. rhamnosus* *Lb. helveticus*), *Streptococcus* (*S. thermophilus*, *S. macedonicus*) and *Leuconostoc* (*Lc. mesenteroides*) (Monterisino et al., 2008; Mozzi et al., 2006; Van der Meulen et al., 2007).

HoPS are composed of repeated units that contain only one type of monosaccharide: D-glucopyranose (glucans) or D-fructopyranose (fructans). These polysaccharides usually display high molecular masses (up to  $10^7$  Da), and have different degrees and types of branching, linking sites and chain length. Based on their structure, the fructans can be divided into two groups: (i) inulins (linked  $\beta$ -2,1) and (ii) levans (linked  $\beta$ -2, 6), both are synthesized by different species of the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Weissella*.

Glucans can be classified into  $\alpha$ - and  $\beta$ -D-glucans. The former are more widely found in LAB and they are produced by strains belonging to the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus*. According to the linkages in the main chain, the  $\alpha$ -glucans are subdivided into dextrans ( $\alpha$ -1,6), mutans ( $\alpha$ -1,3), glucans ( $\alpha$ -1,2), reuterans ( $\alpha$ -1,4) and alternans ( $\alpha$ -1,3 and  $\alpha$ -1,6) (Figure 1). These polymers may have side-chain branches that involve others  $\alpha$ -linkages different from the main chain. For example, the dextrans produced by various LAB

such as *Leuconostoc mesenteroides* and *Lactobacilli*, may have branches with  $\alpha$ -1,2,  $\alpha$ -1,3 or  $\alpha$ -1,4 linkages. The dextran most widely used by the industry is a polysaccharide containing 95%  $\alpha$ -1,6 and 5%  $\alpha$ -1,3 linkages synthesized by *Lc. mesenteroides* NRLL BF512 (Korakli & Vogel, 2006; Monsan et al., 2001; van Hijum et al., 2006).

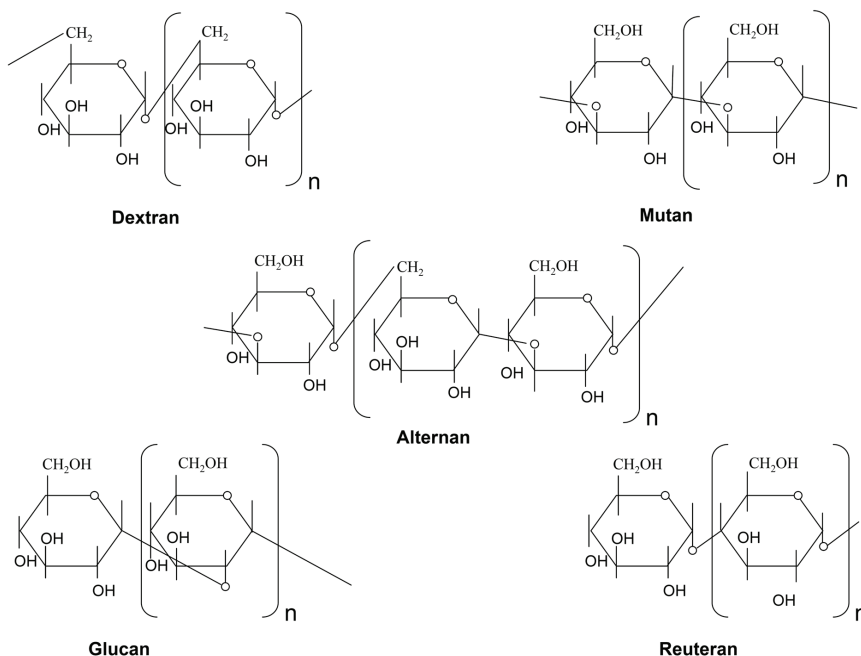


Fig. 1. Schematic representation of the repeating units of: dextran, mutan, alternan, glucan and reuteran (Korakli & Vogel, 2006)

(1,3)  $\beta$ -glucans are found in bacteria and eukaryotic organisms. These polysaccharides include the linear glucans and 6-substituted (1,3)  $\beta$ -glucans that have branch-on-branch or cyclic structures. Concerning prokaryotes, several bacteria including *Agrobacterium* and *Rhizobium* species can produce these polymers. One such structure, curdlan, has been approved as a food additive by the Food and Drug Administration (FDA), and essentially is a linear (1,3)  $\beta$ -D-glucan which may have a few inter- or intra- chain (1,6) linkages (McIntosh et al., 2005).

$\beta$ -glucan production is rarely found in LAB. It has only been reported to be synthesized and secreted by a small number of strains isolated from alcoholic beverages, namely: *Pediococcus parvulus* IOEB8801 and *Oenococcus oeni* IOEB0205 from wine and *P. parvulus* 2.6R, CUPV1, CUPV22, *Lb. diolivorans* G77 and *O. oeni* I4 from cider (Dueñas-Chasco et al., 1997, 1998; Garai-Ibabe et al., 2010; Ibarburu et al., 2007; Llauberes et al., 1990).

In all cases, these  $\beta$ -D-glucans have a common structure comprising a main chain of (1,3)-linked  $\beta$ -D-glucopyranosyl units along with more or less frequent side chains of  $\beta$ -D-glucopyranosyl units attached by (1,2) linkages (Figure 2).

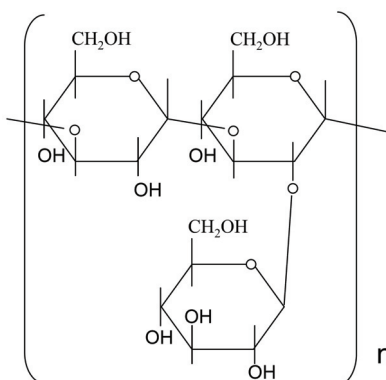


Fig. 2. Schematic representation of the repeating unit of  $\beta$ -(1,3-1,2)-D-glucan of *P. parvulus* 2.6 (Dueñas-Chasco et al., 1997)

In addition, a similar polymer constitutes the capsule of *S. pneumoniae* serotype 37 and the EPS secreted by *Propionibacterium freundenreichii* subsp. *shermanii* TL34 (Adeyeye et al., 1988; Nordmark et al., 2005).

### 3. Biosynthesis of EPS

#### 3.1 Genetic determinants and mechanisms of production and secretion

The genes involved in the biogenesis of the HePS are usually organized in clusters that can be located either in the chromosome of the thermophilic LAB (e. g. *S. thermophilus* Sfi6) or in plasmids of mesophilic bacteria (e.g. *L. lactis* subsp. *cremoris* NIZO B40) (Laws et al., 2001). This structural organization is highly conserved among LAB and is very similar to that observed for the operons and clusters involved in the synthesis of: (i) O-antigen lipopolysaccharides in enterobacteria, (ii) capsules (CPS) of pathogens, such as *S. pneumoniae* or *Staphylococcus aureus* and (iii) the EPS from *Sinorhizobium meliloti* (García et al., 2000; Glucksmann et al., 1993; Lin et al., 1994).

The clusters from LAB have been reported for *S. thermophilus*, *Lb. helveticus*, *L. lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. rhamnosus* and the *Lb. casei* group (Ruas-Madiedo et al, 2008). The genes are oriented in a single direction and transcribed as a single mRNA. The genes are grouped into four regions within the cluster: The first contains genes whose products are regulatory proteins; the second includes genes encoding proteins involved in polymerization/chain length determination; the third contains genes encoding enzymes required for the biosynthesis of the HePS repeating units, and the genes of the last region encode proteins implicated in transport and polymerization (Figure 3) (Jolly & Stinglele, 2001).

Additionally, these clusters may occasionally include genes involved in biosynthesis of nucleotide sugars from which the repeating units are constructed. Thus, in *Lb. rhamnosus*, they are associated to the EPS operon and they can be transcribed either from their own promoter or together with the EPS operon genes (Péant et al., 2005). There is a great variability in the genes involved in the synthesis of the repeating units. This region is

responsible for the production of a specific EPS type; whereas the genes of polymerization and transport are more conserved.

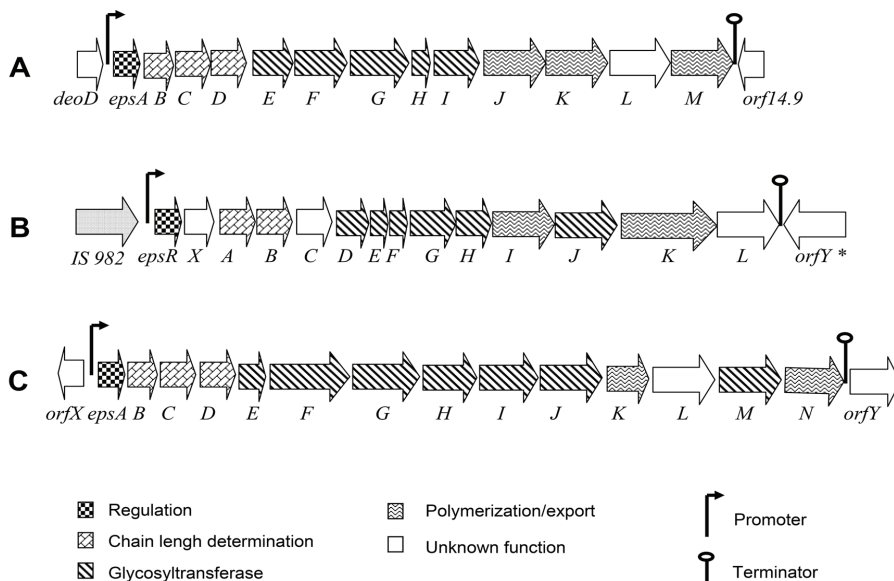


Fig. 3. Organization of the *eps* gene clusters of (A) *S. thermophilus* Sfi6 (B), *L. lactis* subsp. *cremoris* NIZO B40 and (C) *Lb. bulgaricus* Lfi5 (Lamothe et al., 2002; Laws et al., 2001). The proposed function of the different gene products is indicated

HePS are made by the polymerization of repeating unit precursors formed in the cytoplasm. They are assembled at the membrane by the sequential addition of nucleotides sugars (e.g., UDP-glucose, UDP-galactose and dTDP-rhamnose) or nucleoside diphosphate sugars to the growing repeated units through the action of the specific glycosyltransferases. This unit is most probably anchored to a lipid carrier molecule located in the plasma membrane and the first monosaccharide is linked by the action of a type of glycosyltransferase called priming-glycosyltransferase. It has been proposed that this lipid carrier might be an isoprenoid derivative such as undecaprenyl-phosphate (C55-P) by analogy with the synthesis of other EPS of gram-negative bacteria, as well as in the assembly of peptidoglycan, lipoteichoic acids and O-antigen lipopolysaccharides (Ruas-Madiedo et al., 2009). However some studies based in the resistance to bacitracin (a compound that blocks the transformation of the C55-PP to C55-P), suggested that HePS biosynthesis in *S. thermophilus* Sfi6 uses a lipid carrier different from undecaprenyl carrier (Stingle et al., 1999).

The mechanisms of polymerization, chain length determination and export still remain poorly understood. However, the similarity of gene products involved in these processes to those participating in the polymerization and export of O-antigens from *Escherichia coli* and the EPS of *S. meliloti*, suggests that probably LAB utilize similar mechanisms for polymerization and export of EPS. Thus, an enzyme of the flipase family could transfer the lipid-bound repeating units from the cytoplasmic face of the membrane to the external face.

Using the same line of argument, a polymerase could catalyze the linking of the repeating units and an enzyme could uncouple the lipid-bound polymer and control chain length (Laws et al., 2001).

The regulatory mechanisms involved in expression of the HePS genes remain unclear. However, a transcriptional regulatory role has been proposed for a group of gene products in different LAB genera, due to their similarity with a family of transcriptional regulators whose prototype is LytR, the regulator of the autolysin operon of *Bacillus subtilis* (Ruas-Madiedo et al., 2009).

Finally, a variety of nucleotide sugars is needed for the synthesis of a range of polysaccharides which is not specific to EPS biosynthesis. The production of these precursors occurs in the cytoplasm, mainly from glucose 1-P which is synthesized from glucose 6-P. These two forms of phosphorylated glucose are part of the central metabolism of the bacterium, which begins with the transport of sugar to the interior of the cell (de Vuyst et al., 2001).

Concerning to HoPS, most of them ( $\alpha$ -glucans and fructans) are synthesized from sucrose through the action of extracellular enzymes commonly named glycansucrases. Enzymes synthesizing  $\alpha$ -glucan polymers are limited to LAB, while enzymes synthesizing fructans, are present in other gram-positive and gram-negative bacteria. A large number of these glycansucrase encoding genes have been identified in the chromosomes of *Streptococcus*, *Leuconostoc* and *Lactobacillus* strains and they usually form part of a monocistronic transcriptional unit. Some of these genes are expressed constitutively whilst other are sucrose-inducible (Gänzle & Schwab, 2009; van Hijum et al., 2006).

Only one gene, named *gtf*, is required for the  $\alpha$ -glucan biosynthesis in LAB. This gene is located in a 35 kb plasmid of *P. parvulus* 2.6 or in a 5.5 kb plasmid of *P. parvulus* IOEB8801, CUPV1, CUPV22, *Lb. diolivorans* G77 and *Lb. suebicus* CUPV221, while *gtf* is located in the chromosome of *Oenococcus oeni* 14 and IOEB0205 (Dols-Lafargue et al., 2008; Garai-Ibabe et al., 2010; Walling et al., 2001; Werning et al., 2006). All of these bacteria produced the same 2-substitute (1,3)- $\beta$ -D-glucan and their *gtf* genes show high level of homology (at least 97%).

The *gtf* gene encodes a  $\beta$ -glycosyltransferase (GTF). It is a membrane protein whose topological prediction indicates that  $\beta$ -glucan or, at least its repeating unit precursors, are synthesized in the cytosol (Werning et al., 2006). In agreement, *in vitro* experiments indicate that the  $\beta$ -glucan polymer is synthesized by GTF directly from UDP-glucose (Werning et al., 2008) which, as mentioned above, is part of the central metabolism of the bacterium and is available within the cell. The translocation through the membrane is performed by a mechanism that is not yet known. The fact that heterologous expression of GTF in other LAB leads to the synthesis and secretion of  $\beta$ -glucan in the recipient bacteria, strongly suggests that this polymer does not require specific transporters to be released into the extracellular space (Stack et al., 2010; Werning et al., 2008). Supporting this hypothesis, the capsular 2-substituted (1,3)- $\beta$ -glucan of *S. pneumoniae* serotype 37, is synthesized by a single  $\beta$ -glycosyltransferase called Tts (which shares a 33% identity with GTF), and its heterologous expression in other gram-positive bacteria, also result in the capsular EPS formation (Llull et al., 2001).

The presence of four potential transmembrane regions at the C-terminal region of GTF glycosyltransferase and two more at its N-terminus suggest the formation of a membrane pore by the enzyme to facilitate the extrusion of the polymer (Werning et al., 2006). In this regard, there is experimental evidence (Helderman et al., 2001; Tlapak-Simons et al., 1998, 1999) suggesting that in the HAS glycosyltransferase from *S. pyogenes* only four transmembrane domains and two membrane-associated regions are sufficient to interact with the membrane phospholipids and to create a pore-like structure through which a nascent hyaluronan chain can be extruded to the extracellular environment. A similar mechanism could happen in the translocation of curdlan through the *Agrobacterium* membrane, for which it has been proposed that a pore could be formed by interaction of the CrdS synthase with phosphatidylethanolamine (Karnezis et al., 2003). Thus, the association of several GTF monomers could form a pore for extrusion of the EPS and, that might promote the processive catalysis of GTF carried out by this enzyme. However other alternative mechanisms might allow the translocation or secretion of  $\beta$ -glucan across LAB membranes. This includes the use of an ABC-like transporter, since this class of transporter can export various bacterial polysaccharides (Silver et al., 2001).

Finally, it should be noted that the presence of mobile genetic elements is a common feature in the genetic organization of the DNA region involved in HePS or HoPS synthesis (Bourgoin et al., 1999; Dabour & LaPointe, 2005; Peant et al., 2005; Tieking et al., 2005; van Hijum et al., 2004). It is well known that these elements allow horizontal transfer between different genera. In this regard, its presence could explain the instability of the HePS-producer phenotype of some strains (Ruas-Madiedo et al., 2009) as well as the loss of expression of some glycansucrases or the presence of chimeras in different HoPS-producing strains (Gänzle & Schwab, 2009). The *gtf* genes of LAB are flanked by genes which could be involved in functions of conjugation and recombination, respectively (Werning et al., 2006; Dols-Lafargue et al., 2008). Thus, horizontal transfer mediated by plasmids or transposition events, might explain the wide distribution and high degree of *gtf* gene preservation in  $\beta$ -glucan-producing strains belonging to different genera.

### 3.2 Enzymes for production.

HePS and  $\beta$ -glucans are produced by glycosyltransferases which use sugar nucleotides as substrate. On the other hand,  $\alpha$ -glucans and fructans are synthesized by glycansucrases which are able to use the energy of the glycosidic bond of sucrose to ligate glucose or fructose to the growing polysaccharide chain. In addition, these enzymes can synthesize hetero-oligosaccharides, when the acceptors are maltose and isomaltose (Monsan et al., 2001).

Enzymes synthesizing  $\alpha$ -glucan polymers are called glucansucrases (GS) and those synthesizing fructans are named fructansucrases (FS). Unlike the glycosyltransferases (discussed below), GS and FS are transglycosidases evolutionarily, structurally, and mechanistically related to the glycosyl-hydrolases (GH). Therefore, according to the classification of the GH into families (which is based on the amino acid sequences) GS and FS can be respectively placed within the GH70 and the GH68 families (Henrissat & Bairoch, 1996). In addition, according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), GS enzymes are classified (according to the reaction they catalyze and the type of product) into: (i) dextransucrases (E.C.2.4.1.5) and (ii)

alternansucrases (E.C.2.4.1.140). The mutansucrases and reuteransucrases are classified together with the dextransucrase enzymes. Also, FS enzymes can be classified, based on the different products synthesized, into: (i) inulosucrases (E.C.2.4.1.9) and (ii) levansucrases (E.C.2.4.1.10) (van Hijum et al., 2006).

Although GS and FS enzymes perform very similar reactions on the same substrate, they do not show great similarities in their amino acid sequence and strongly differ in their protein structures. Regarding the amino acid sequence, these enzymes are composed of 4 structural domains from the N to the C terminus: (i) a signal peptide involved in the secretion of the enzyme; (ii) a variable N-terminal domain of unknown function; (iii) a conserved catalytic domain comprising sucrose-binding and sucrose-cleaving domain and in FS also a calcium ion binding site and (iv) a C-terminal domain, that is composed of a series of tandem repeats which is thought to be involved in the control of product size as well as in  $\alpha$ -glucan binding (GS), or in cell wall anchoring (FS) (Korakli & Vogel, 2006).

The mechanism of action of GS has still not fully understood. The key step in the transfer of D-glucosyl units is the formation of a covalent glucosyl-enzyme intermediate, in which an amino acid triad, composed of two aspartate and one glutamate residues, is involved. From this intermediate, the glucosyl unit is transferred to the acceptor (the polymer in growth) by a processive catalytic mechanism. The overall synthesis can be described as three steps: initiation, elongation and termination. The last corresponds to the dissociation of the  $\alpha$ -glucan-enzyme complex. Regarding the elongation, this can proceed by two alternative mechanisms, one acts at the reducing end and the other at the non-reducing end of the growing  $\alpha$ -glucan chain (Monchois et al., 1999; Monsan et al., 2001).

So far little is known about the mechanism of action of the FS enzymes. Fructan biosynthesis could be carried out by a multiple elongation mechanism, where the fructose residues are added to the growing fructan chain. The catalytic mechanism proposed for the transfructosylation reaction occurs in two steps, involving both a nucleophilic and an acidophilic site, through the formation of a covalently linked intermediary fructosyl-enzyme (Monsan et al., 2001; Sinnott, 1991).

The glycosyltransferases are ubiquitous enzymes in prokaryotes and eukaryotes. They are involved in the biosynthesis of oligosaccharides, polysaccharides and glycoproteins (e.g. lipopolysaccharides and glycoproteins). These enzymes are responsible for the biosynthesis of glycosidic bonds by the transfer of a sugar residue from activated donor molecules to specific acceptor molecules. Donor sugar substrates are mostly nucleotide sugars; however they can also be nucleoside monophosphate sugars, lipid phosphate sugars and sugar 1-phosphate. Frequently the acceptors are other sugars but they can be lipids, nucleic acids, antibiotics, etc. Additionally, two stereochemical outcomes are possible for reactions that result in the formation of a new glycosidic bond: the anomeric configuration of the product can be retained ( $\alpha$ -glycosyltransferases) or inverted ( $\beta$ -glycosyltransferases) with respect to the donor substrate (Lairson et al., 2008).

The HePS biosynthesis in LAB involves several enzymes for production of repeating units: a *priming*-glycosyltransferase that transfers the first sugar from sugar 1-phosphate onto a phosphorylated carrier lipid and one or more  $\alpha$ - or  $\beta$ -glycosyltransferases that sequentially add new sugars from nucleotide sugars to the growing repeating unit. Some already characterized examples of these enzymes are: EpsE, a phospho-galactosyltransferase from *S.*



*thermophilus* Sfi6 and EpsD a phospho-glucosyltransferase from *L. lactis* subsp. *cremoris* B40, which are priming-glycosyltransferases. Examples of other glycosyltransferases are: EpsG, an N-acetyl-glucosaminetransferase from *S. thermophilus* Sfi6, which transfers N-acetylglucosamine to a  $\beta$ -galactose precursor anchored to a carrier lipid or EpsG, that catalyzes the linkage of galactose to a cellobiose precursor anchored to a carrier lipid from *L. lactis* subsp. *cremoris* (Ruas-Madiedo et al., 2009).

As mentioned above, the 2-substituted (1,3)- $\beta$ -D-glucan biosynthesis of *P. parvulus* 2.6 is carried out by the GTF glucosyltransferase. GTF was overproduced in *L. lactis* NZ9000 and purified as a membrane-associated enzyme (Werning et al., 2008). These membrane preparations used UDP-glucose as donor substrate to catalyze the biosynthesis of a high molecular weight polysaccharide that corresponds to the 2-substituted (1,3)- $\beta$ -D-glucan (Werning et al., 2008 and unpublished results). The acceptor is so far unknown, but it could be the growing  $\beta$ -glucan polymer or any lipid molecule present in the cellular membranes.

Traditionally, glycosyltransferases have been classified on the basis of their donor, acceptor and product specificity according to the recommendations of the IUBMB. However, this system requires full characterization before an Enzyme Commission (EC) number can be assigned. To overcome this limitation, these enzymes have been classified into families on the basis of amino acid sequence similarities as in the case of GH. At present there are already 91 families (referred to as GTx), available at URL: [http://www.cazy.org/fam/acc\\_GT.html](http://www.cazy.org/fam/acc_GT.html). This classification is periodically reviewed and updated (Coutinho et al., 2003).

According to protein sequence similarity, enzymes that produce HePS can be grouped into various families. With regard to HoPS, the dextransucrases have been included in the family of glycosyl hydrolases GH70. In addition, GTF belongs to the GT-2 family that includes other glycosyltransferases such as cellulose synthases,  $\beta$ -1,3 glucan synthases, chitinsynthases, HAS and  $\beta$ -glucosyltransferases. All of these enzymes have in common that they use a nucleotide sugar as substrate for the synthesis of a polymer, with inversion of anomeric configuration of the donor substrate.

Two general three-dimensional (3D) folding models, called GT-A and GT-B have been observed for all the structures of nucleotide-sugar-dependent glycosyltransferases solved to date, and two mechanisms for retaining or inverting enzymes can be proposed within both classes. The GT-A fold may be considered as two tightly associated and abutting  $\beta/\alpha/\beta$  Rossmann domains that tend to form a continuous central sheet of at least eight  $\beta$ -strands. The GT-B fold consists of two  $\beta/\alpha/\beta$  Rossmann domains that face each other and are linked flexibly. These domains correspond to the donor substrate and acceptor binding sites (Breton et al., 2005; Lairson et al., 2008).

So far, no 3D-structure has been resolved for any glycosyltransferase involved in EPS synthesis from LAB, which would be essential for a better understanding of the mode of action of these enzymes. However, using the GT classification system proposed by Coutinho et al (2003) (in which families can be classified into clans on the basis of their folding and stereochemical outcome of the reaction that they catalyze) it is possible to predict that the glycosyltransferases can adopt one of two possible foldings. Thus, GTF from *P. parvulus* 2.6 belongs to the inverting-clan I of GT-A glycosyltransferases. A 3D-dimensional model based on the sequence of the putative active domain of GTF was built using as template the

experimentally resolved structures of SpsA synthase from *Bacillus subtilis* (PDB code 1qgq) and of a putative glycosyltransferase from *Bacteroides fragilis* (PDB code 3bcv). The model proposed for GTF corresponds to a GT-A fold, which consist of an open twisted  $\beta$ -sheet surrounded by  $\alpha$ -helices on both sides, where the N- and C-terminus domains are respectively the donor substrate and the acceptor binding sites (Figure 4).

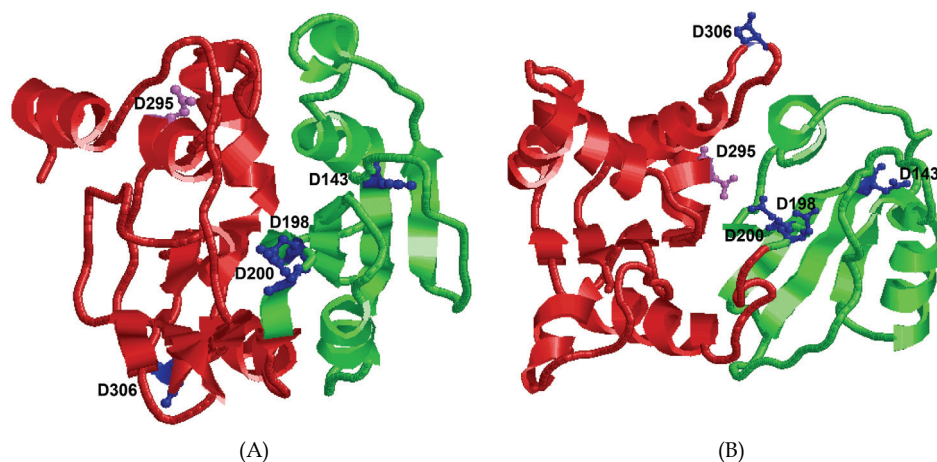


Fig. 4. Three-dimensional model for the putative active site of GTF constructed using resolved structures of SpsA from *B. subtilis* (A) and a putative glycosyltransferase from *Bc. fragilis* (B). The conserved N-terminal nucleotide-binding domain is shown in green and the C-terminal acceptor domain in red. The ball-and-stick representations show putative residues involved in the catalytic center: D143, D198 and D200 at the N-terminal domain and D306 at the C-terminal domain (in blue) as well as D295 (in violet), which is an alternative residue to D306, whose putative functions are discussed in the text

Based on structural data and on site directed mutagenesis experiments, it has been proposed that only four aspartic or carboxylate groups are required to form a single catalytic center of GT-A inverting enzymes (Charnock et al., 2001; Garinot-Schneider et al., 2000; Keenleyside et al., 2001; Tarbouriech et al., 2001). Three Asp located at the N-terminus domain are involved in nucleoside diphosphate (NDP) coordination. The first Asp residue is implicated in the recognition of uracil or thymine base. The other two, commonly referred to as DXD motif, bind to hydroxyl groups on the ribose moiety and the divalent metal ion ( $Mg^{2+}$  or  $Mn^{2+}$ ), which in turn coordinate the phosphate groups from NDP, facilitating the release of the NDP from the donor substrate. A fourth residue, Asp or Glu, in the acceptor domain (C-terminus) acts at the catalytic site by activating the acceptor hydroxyl group, which would subsequently perform a nucleophilic attack on the C1 of the donor substrate (Lairson et al., 2008).

Candidate aspartate residues exist in GTF and it is possible to predict their location in the active site of this protein based on the 3D-structural model shown in Figure 4. Three aspartates (D143, D198 and D200) are coincident with those conserved and proposed in other glycosyltransferases from the GT-2 family, including glycosyltransferase SpsA of *B. subtilis*. The fourth aspartate (D306) is not a good candidate to be part of the active site

according to the current models and one alternative could be that at D295, though this should be evaluated by substrate docking experiments and site directed mutagenesis.

## 4. Production, purification and analysis of EPS

### 4.1 Methods of production

Most LAB species show low yields of polysaccharide production which is the main reason for their lack of commercial exploitation. Generally (with a few exceptions) the yield of production is under 1 g L<sup>-1</sup> for HoPS, when culture conditions are not optimized, and even less for the majority of the HePS. Van der Meulen et al. (2007) reported the EPS production by 10 LAB strains isolated from dairy and cereal products. Nine out of ten produced glucans in amounts from 0.8 to 1.7 g L<sup>-1</sup>. The only HePS producer was *Lb. curvatus*, which synthesized the EPS to levels of 22 mg L<sup>-1</sup>. Mozzi et al. (2006) reported that 31 HePS producers screened from 201 LAB strains (including thermophilic and mesophilic species) synthesized from 10 to 166 mg L<sup>-1</sup>. Only seven of them produced > 100 mg L<sup>-1</sup>.

Efforts have been made to improve yields of EPS production by LAB resulting in a variety of methods well detailed in the literature. All of them focus on parameters that have a strong influence on the production of HoPS and HePS. As a general rule the amount and composition of the EPS produced by LAB is strongly influenced by the culture and fermentation conditions such as pH, temperature and medium composition (Dueñas et al., 2003).

The production of  $\alpha$ -glucans by LAB can be obtained in the presence of sucrose, after optimization of sucrose concentration in the growth medium and the time of incubation. The depletion of the sucrose source would cause the arrest of the enzymatic reaction of the dextranases. It has been reported that high producers of dextran are primarily *Leuconostoc* strains, but many other strains of LAB are able to produce this bacterial polysaccharide (Sarwat et al., 2008). The importance of improving its production is related to the industrial applications in the food, pharmaceutical and chemical industries as adjuvant, emulsifier, carrier and stabilizer (Goulas et al., 2004). *Lc. mesenteroides* CMG713 produces the highest concentration of dextran after 20 hours of incubation at 30°C in the presence of 15% sucrose at pH 7.0, with an EPS yield of 6 g L<sup>-1</sup> (Sarwat et al., 2008). Recently, Capek et al. (2011) reported an exceptionally high production of this HoPS (50 g L<sup>-1</sup>) by *Lc. garlicum* PR.

On the contrary,  $\beta$ -glucan production is very tedious, because of the low yield obtained. Therefore, it demands new strategies to improve synthesis apart from the optimization of growth parameters. For production of the 2-substituted (1,3)  $\beta$ -glucan heterologous gene expression has been tested. A plasmid, pNGTF, was constructed in order to express the *P. parvulus* *gtf* in *L. lactis* NZ9000. This plasmid allows inducible expression of the *gtf* gene from the *nisA* gene promoter by the addition of nisin to the growth medium (Werning et al., 2008). The EPS released to the medium by NZ9000[pNGTF] was quantified and purified. The expression of GTF glycosyltransferase by NZ9000[pNGTF] yielded levels of purified EPS of 300 mg L<sup>-1</sup>, when the bacteria was grown in batch conditions (Werning et al., 2008). The structural characterization of the purified EPS confirmed that the recombinant strain synthesizes and secretes the same 2-substituted (1,3)- $\beta$ -D-glucan as *P. parvulus* 2.6 (Werning et al., 2008). The synthesis of the EPS was still not very high, but it could probably be

improved by optimizing growth conditions of the producing recombinant strain in continuous culture in a chemostat at controlled pH.

With regard to HePS, their production has been improved in the native isolates by optimizing growth conditions and media composition. As an example, Vijayendra and Babu (2008) optimized the EPS production by *Leuconostoc* sp. CFR-2181, a strain isolated from dahi, an Indian traditional fermented dairy product, in a simple, low cost semisynthetic medium. Maximum biomass and HePS production was observed when sucrose was used as carbon source and a yield of 30 g L<sup>-1</sup> of the biopolymer was obtained, although the concentration was estimated by dry weight and purity of the HePS was not assayed. As an alternative, to improve production of HePS, the *eps* clusters of various LAB have been overexpressed, mainly in *L. lactis*, by construction of recombinant strains carrying the genes in multicopy plasmids (e.g. the HePS of *S. thermophilus* Sfi39). In addition, metabolic engineering of *L. lactis* has been used to redirect carbon distribution between glycolysis and sugar nucleotide biosynthesis, with the aim of increasing intracellular levels of UDP-glucose, UDP-galactose and UDP-rhamnose, the substrates of the glycosyltransferases encoded by plasmid pNZ4000 and involved in HePS biosynthesis in the NIZO B40 strain (Boels et al., 2002).

The importance that EPS has gained in the food industries has been responsible for the development of other strategies to improve the total amount produced. Some good examples are their *in situ* production in food matrices and their *in vitro* production by use of immobilized enzymes.

LAB can produce a large variety EPS during elaboration of dairy products. Since the use of LAB is historically considered safe (GRAS microorganisms), production *in situ* of novel functional EPS means that toxicological testing will be reduced, or not required, and the products can be quickly brought to the market (De Vuyst et al, 1999).

Yogurt is a well-known dairy product derived from milk fermentation by cultures producing EPS (e.g. *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* produce, respectively, 60-150 mg L<sup>-1</sup> and 30-890 mg L<sup>-1</sup> of HePS) (Marshall and Rawson, 1999). The use of EPS-producing starter cultures for yogurt elaboration is increasing, because these biopolymers improve water retention and texture and confer thickness without altering the organoleptic characteristics of the final product. Thus, there is no need to add stabilizers, many of which are prohibited in a wide range of countries. Although the role of pure EPS has not been studied, authors agree that the key points of improving the texture are the conformation of the EPS and their interactions with casein. (Badel et al., 2011).

In the cheese making process strains such as *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. helveticus* and *Lb. casei*, produce HePS. These polysaccharides, as occurs in yogurt, help to improve the rheological properties of the cheese. Their role in cheese elaboration depends on associations with other strains and also on the presence or absence of charges in the EPS produced. (Badel et al., 2011).

Furthermore, there are other examples of *in situ* EPS production, such as kefir. This is a very important beverage in Eastern European countries. It is a fermented milk product produced by a population of different species of bacteria and yeasts. Several functional properties of kefir, such as is ability to modulate immune responses, to diminish allergic reactions and to inhibit tumour growth has been postulated for this beverage (Liu et al., 2002). LAB produce

lactic acid and yeast synthesize ethanol, which is used by *Lb. kefiranofaciens* to produce kefiran, a polysaccharide resistant to enzymatic degradation. Kefiran is a natural biopolymer that could be used as a thickener in fermented products.

Finally, the use of enzymes for the *in vitro* synthesis of polysaccharides is showing promise. Immobilized enzymes are preferred as this allows the recovery and reuse of the enzyme, and may improve the properties of the enzyme such as stability, activity, specificity and selectivity (Mateo et al. 2007). The technique is used for production of isomaltooligosaccharides by immobilized dextranase. Isomaltooligosaccharides are oligosaccharides with prebiotic activity that can be produced either by acceptor reactions of dextranase or hydrolysis of dextran by dextranase. In the case of dextranase, it can also produce leucrose (a disaccharide used as sweetener). Other uses are also currently being developed e.g. immobilization of  $\beta$ -galactosidase to produce galactooligosaccharides. Concerning immobilization technology, the alginate encapsulation method has the best performance, rendering yields of up to 90% (Tanriseven & Dogan, 2002). Dextranase has been immobilized on various supports including glutaraldehyde-activated chitosan, porous glass, bentonite and the commercially available matrix, Eupergit C, with high yield (90%) (Aslan & Tanriseven, 2007). Dextranase and dextranase share the optimum pH (pH 5.4) which facilitates their combined use. However, few studies using co-immobilization of dextranase and dextranase are yet available (Frank et al. 2008; Olcer & Tanriseven, 2010).

## 4.2 Methods of purification and characterization of EPS

Purification is the physical separation of a chemical substance of interest from contaminating substances. In the case of EPS purification from bacterial culture supernatants means elimination of producer microorganisms and their secreted metabolites as well as components of the growth media. Ruas-Madiedo et al. (2005) extensively reviewed this subject, thus we shall present here only an overview of the more usual procedures, with a more detailed description of methods related to the determination of EPS structure.

The first step of purification of EPS depends on the bacterial growth medium utilized for its production. In complex media or in food matrix, such as milk, the first requirement is the elimination of proteins. For their removal a precipitation with TCA as well as treatment with proteases are the most commonly used methods. Then, the supernatant as well as the supernatant of bacterial cultures grown in defined media are usually subjected to one or more cycles of precipitation with either ethanol or acetone. The biopolymers present in the supernatants, if they are soluble, are dissolved in water, and then dialysed to remove the low molecular weight contaminants, in general a membrane with a cut-off of MWCO 12-14.000 Da is used.

After lyophilisation of the samples, the EPS is often further purified using a chromatographic technique. The parameters involved in the choice of the appropriate chromatography are: charge, solubility and molecular weight of the EPS. Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, not by molecular weight. It is usually applied to large molecules or macromolecular complexes. Another example is ion-exchange chromatography, which

allows the separation of ions and polar molecules based on their charge. In general the procedure involves passing a mixture dissolved in a mobile phase through a stationary phase. For high molecular weight polysaccharides, such as the 2-substituted-(1-3)- $\beta$ -glucan, SEC is the most suitable method, because the charge of the EPS is zero. In this case, dried EPS are dissolved in 0.3 M NaOH (to eliminate extra contaminants and to improve the solubility of the EPS) and centrifuged to eliminate insoluble material. The supernatant is loaded into a column of Sepharose CL-6B equilibrated with NaOH, which is also used as eluent. Fractions are collected, and monitored for carbohydrate content by the phenol-sulphuric method (DuBois et al., 1956).

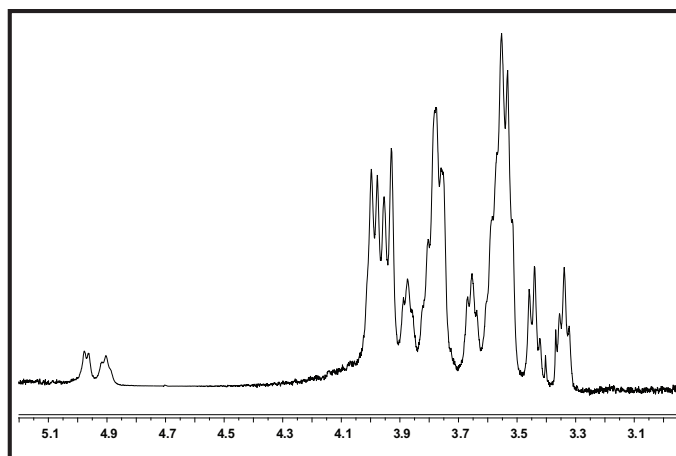
Polysaccharides are polydisperse polymers, and consequently only an apparent average molecular weight ( $M_w$ ) can be determined. To this end, the average  $M_w$  can be estimated after SEC fractionation. A calibration curve is performed by fractionation of standards (Dextran Blue, T70, T10, and vitamin B12) and used for the determination of the  $M_w$ . As alternative, high-performance size-exclusion chromatography (HPSEC) equipped with multi-angle laser-light scattering (MALLS) and refractive index (RI) detectors can be used to determine weight-average molecular weight ( $M_w$ ) and z-average radius of gyration ( $R_z$ ) of the EPS.

To determine the monosaccharide composition of the EPS, the analysis of neutral sugars is performed by polysaccharide hydrolysis with 3M TFA. The resulting monosaccharides are converted into their corresponding alditol acetates by reduction with  $\text{NaBH}_4$  and subsequent acetylation (Laine et al., 1972). Identification and quantification is performed by gas-liquid chromatography (GLC) using a HP5 fused silica column, with a temperature program and a flame ionization detector. With this technique the chromatogram shows only one peak per each monosaccharide, leading to an easy identification of the monosaccharide composition. However, if the polysaccharide contains uronic acid(s) it must be subjected to methanolysis after the hydrolysis. An *O*-methyl glycoside is formed, the acid function is transformed into an ester group and the sugar derivative can then be acetylated and analyzed by GLC.

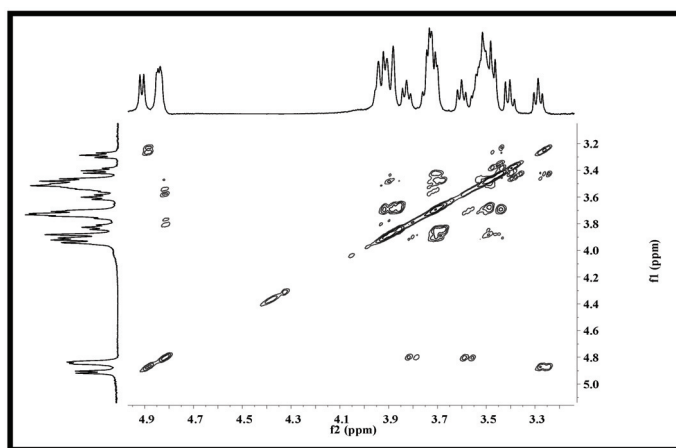
To determine the type of bond between each residue present in the EPS molecule a methylation analysis is usually performed. The polysaccharides are methylated according to the method of Ciucanu and Kerek (1984). The partially methylated polysaccharides are hydrolyzed with 3M TFA and the products are reduced with  $\text{NaBD}_4$ , acetylated and analyzed by gas chromatography/mass spectroscopy (GC-MS) (Leal et al., 2008). Each peak of the chromatogram is identified by the retention time and mass spectra parameters. The quantification is associated to the peaks area.

To resolve the 3D-structure of an EPS molecule, both the ring size (pyranose/furanose) of the monosaccharide residues and the relative orientations of the adjacent monosaccharides have to be determined. Nuclear magnetic resonance (NMR) is the technique most often used to study the conformation of the polysaccharides and allows elucidation of the type of glycosidic linkages and the structure of the repeating units that constitute the EPS molecules (review by Duus et al., 2000). Before NMR analysis, the purified EPS is dissolved in  $\text{D}_2\text{O}$  so that exchangeable protons are replaced by deuterium (deuteration). This procedure is repeated several times and may involve intermediate lyophilisation steps. A  $^1\text{H}$  NMR spectrum of the EPS gives information about the number of monosaccharide present in the

repeating unit by counting the resonances in the anomeric region (4.4-5.5 ppm). The common hexoses are detected as well in  $^{13}\text{C}$  NMR spectra (95-110 ppm). If there are resonances just downfield of 1 ppm in the  $^1\text{H}$  NMR spectrum it is a sign of  $\text{CH}_3$ -groups of e.g. a fucose or a rhamnose residue. Resonances close to 2 ppm reveal *N*-acetyl and/or *O*-acetyl functionalities. From the splitting of the anomeric peaks in  $^1\text{H}$  spectra ( $J_{\text{H1,H2}}$ ) the anomeric configuration can be established; a *J*-coupling of  $\sim 4$  Hz indicates the  $\alpha$ -configuration and a value of  $\sim 8$  Hz indicates the  $\beta$ -form for common monosaccharides like D-glucopyranose and D-galactopyranose. The corresponding values of  $J_{\text{C1,H1}}$  are  $\sim 170$ -175 Hz for the  $\alpha$ -form and  $\sim 160$ -165 Hz for the  $\beta$ -form obtained from a coupled  $^{13}\text{C}$  NMR analysis. Since most polysaccharide NMR spectra show peak overlap in the ring region ( $\delta_{\text{H}}$



(A)



(B)

Fig. 5. NMR analysis of the purified the 2-substituted (1,3)- $\beta$ -D-glucan.  $^1\text{H}$ -NMR (A) and  $^1\text{H},^1\text{H}$ -COSY (B) spectra are depicted

3.1-4.4), 2D-NMR techniques are often applied. The proton chemical shifts are linked to their respective carbon by a  $^1\text{H},^{13}\text{C}$ -HSQC NMR analysis or, when the resonances in the  $^{13}\text{C}$  dimension overlap too much, a  $^{13}\text{C},^1\text{H}$ -HETCOR NMR analysis is performed. To distinguish methylene protons ( $\text{CH}_2$ ) from methyl ( $\text{CH}_3$ ) and methine ( $\text{CH}$ ), a multiplicity-edited  $^1\text{H},^{13}\text{C}$ -HSQC analysis can be used. The protons in each spin system can be assigned using  $^1\text{H},^1\text{H}$ -TOCSY and/or  $^1\text{H},^1\text{H}$ -DQF-COSY analysis, both techniques allow the magnetization to travel over bonds with the help of  $J$ -couplings and thereby connecting the protons. EPS contain protons and carbons, but sometimes also nitrogen and even phosphorus. Their respective chemical shifts can be assigned and correlated to  $^1\text{H}$  using e.g.  $^1\text{H},^{15}\text{N}$ -HSQC and  $^1\text{H},^31\text{P}$ TOCSY analysis. The exchangeable amide protons, measured in a  $\text{H}_2\text{O}-\text{D}_2\text{O}$  mixture, have  $J$ -couplings to the ring protons and can be assigned correctly in the ring by a  $^1\text{H},^1\text{H}$ -TOCSY with water suppression. As an example, the uni- and bi-dimensional NMR analysis of the 2-substituted (1,3)- $\beta$ -D-glucan is depicted in Figure 5. The 1D-NMR spectrum (Fig. 5A) revealed several peaks in the region between 3.2 and 4.1 ppm and 2 peaks in the anomeric region characteristics of this type of polysaccharide. 2D-COSY analysis (Fig. 5B) showed couplings between anomeric protons and C2 protons ( $\text{H}_{1\text{C}}/\text{H}_{2\text{C}}$ ,  $\text{H}_{1\text{B}}/\text{H}_{2\text{B}}$  y  $\text{H}_{1\text{A}}/\text{H}_{2\text{A}}$ ) characteristic of the 2-substituted (1,3)- $\beta$ -D-glucan.

Finally, to determine the supramolecular structure and conformation of an EPS, atomic force microscopy (AFM) is currently used. It has been applied successfully to visualize a range of polysaccharides including curdlan (Ikeda & Shishido, 2005), and oat  $\beta$ -glucan (Wu et al., 2006). The (1,3)- $\beta$ -D-glucan helices dissociate into random coils when the strength of the bonds keeping the helix together are decreased below a critical limit. The helix-coil transition is referred to as denaturation (Sletmoen & Stokke, 2008). Denaturation of (1,3)- $\beta$ -D-glucan triplexes occurs in alkaline solutions or in dimethyl sulfoxide. In alkaline solutions due to the ionization of the hydroxyl groups and the subsequent electrostatic repulsion between chains, a previous dissociation of the aggregates takes place and then, as the alkalinity increases, the helix structure is believed to denature to yield individual disordered single chains (Sletmoen & Stokke, 2008).

## 5. Biotechnological applications of EPS from LAB

### 5.1 Current applications of the EPS in the food industry

High molecular weight polysaccharides are used as additives in the manufacture of a wide variety of food products, because they act as thickeners, stabilisers, viscosifiers, emulsifiers or gelling agents. Most of these polysaccharides are derived from plants (e.g. pectin, cellulose) and seaweeds (e.g. alginate, carrageenan) (Kleerebezem et al., 1999). In terms of applications in the food industry, microbial extracellular polysaccharides including HePS such as xanthan from the phytopathogenic bacterium *Xanthomonas campestris* and gellan from *Sphingomonas paucimobilis*, are also alternative sources of biothickners approved by the FDA for use as foods additives (Laws et al., 2001). Although these are prepared in reliable quantities, their physical properties might not suit all applications, given that there is also a demand for novel materials that can improve rheological characteristics and health promoting properties. On the other hand, the use of bacterial polysaccharides as food additives requires their production by non-pathogenic bacteria. In this sense, LAB have QPS (qualified presumption of safety) status and EPS produced by these bacteria can be considered as food-grade additives (Ruas-Madiedo et al., 2008).



LAB are routinely used in food preparations, not only due to their metabolic activities, but also due to their preservative effects such as: (i) acidification or production of hydrogen peroxide and (ii) the production of bacteriocins (e.g. nisin), which restrict microbial contamination (Kuipers et al., 1998; Wood, 1997). In addition, EPS production by LAB has received considerable attention, since they provide thickening properties and contribute to improve the texture and mouth-feel of the resulting fermented milks or other dairy products. Moreover, certain EPS produced by LAB, have beneficial effects on human health such as cholesterol-lowering, immunomodulation and prebiotic effect, features that are discussed later. It is therefore considered an advantage to use these polymers as food additives rather than the gram-negative polymers, for which no health promoting abilities have been proposed (Ruas-Madiedo et al., 2008).

EPS may act both as a texturizer, improving the rheology (viscosity and elasticity) of a final product, and as physical stabilizers by binding hydration water and interacting with other milk constituents (ions and proteins) thus limiting syneresis. These physical and rheological properties depend on features such as chemical composition, molecular size, charge, presence of side chains, rigidity of the molecules and 3D- structures of the EPS polymers. In addition to physical characteristics, the interactions between EPS and various components in food products contribute to the development of the final product. Nevertheless, many studies have shown that rheological properties of fermented milk products do not correlated well with the amount of EPS content (de Vuyst, et al., 2001; Duboc & Mollet, 2001; Folkenberg et al., 2006).

Despite the above, EPS from LAB have not yet been exploited industrially as food additives and one of the main drawbacks to use these polymers for such purpose is the low production level compared with xanthan (de Vuyst & Degeest, 1999). Furthermore, low cost culture media and easy isolation procedures, both rendering high yields are essential for the application of EPS as food grade additives. For this reason, production *in situ* by LAB can be an alternative to the use of biopolymers from plants or non-GRAS bacteria. In particular, HePS producing LAB are used in the dairy industry, mostly belonging to the genera *Streptococcus*, *Lactobacillus* and *Lactococcus* to improve the texture and organoleptic properties of the product. Some examples of these are the production of fermented milks such as viili and langmjolk in Nordic countries as well as the production of kefir, yogurt and low fat cheese type mozzarella and Cheddar cheese (de Vuyst et al., 2001; Ricciardi & Clementi, 2000; Ruas-Madiedo et al., 2008). Another application in development for the bakery industry is the *in situ* production of glucans or fructans by the use of *Lactobacillus* or *Weissella* strains in sour dough manufacture (Tieking et al., 2003).

The use of cereal-base substrates is considered as a promising alternative to fermented dairy products due to their high nutritional value and the presence of both soluble and insoluble dietary fiber (Angelov et al., 2005; Materson et al., 2005). In this sense, regarding the development of new functional foods, and their particular ability to produce 2-substituted (1,3)-  $\beta$ -glucans, *P. parvulus* 2.6 and *Lb. diolivorans* G77 have been studied as starter cultures in the preparation of oat-based fermented foods. It has been found that these bacteria can grow and produce the EPS in the oat-base substrate, improving the viscosity and texture of the fermented product (Martensson et al., 2003). In addition, analysis of the rheological properties of the  $\beta$ -glucan synthesized by *P. parvulus* 2.6 showed that it has potential utility as a biothickener (Velasco et al., 2009). Also, it has been reported that differences in the

viscosity of two cultures of different strains of *Pediococcus parvulus* were not attributable to differences in the primary structure or molecular mass of the  $\beta$ -glucan produced (Garai-Ibabe et al., 2010). Other factors such as EPS conformation or interactions between EPS and growth media microstructure could also affect the rheological features. Thus, presumably in the near future, 2-substituted (1,3)-  $\beta$ -glucan-producing LAB will be the used for elaboration of non-dairy fermented food. Moreover, the  $\beta$ -glucan could be used as a food additive, due to its gelling properties.

## 5.2 Potential applications of EPS for production of functional food

### 5.2.1 Prebiotics

The concept of prebiotic was originally defined as “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (Gibson & Roberfroid, 1995). Nine years later, this definition was revised by Gibson et al. (2004) and redefined the concept of prebiotic as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health”.

The non-digestible oligosaccharides (NDO) are the prototypes of prebiotic saccharides. The oligosaccharides are compounds with lower molecular weight due to a lower degree of polymerization (DP). Although the IUB-IUPAC defined oligosaccharides as saccharides composed of 3 to 10 monosaccharide units, other sources define them as compounds with 3 to 20 monosaccharide units. Since, there is not a standard definition, the use of “short-chain carbohydrates” as a term to include oligosaccharides and smaller polysaccharides seems to be more appropriate. The NDO are oligosaccharides with monosaccharide units, having a configuration that makes their osidic bonds non-digestible by the hydrolytic activity of the human digestive enzymes (Roberfroid & Slavin, 2000). They have a low calorific value, non-cariogenicity, are associated with a lower risk of infections and diarrhoea, promote the growth of beneficial bacteria in the colon and an improvement of the immune system response (Mussatto & Mancilha, 2007). The ability of the gut microbiota to ferment oligosaccharides depends on a variety of factors including the degree of polymerization, type of sugar, the glycosidic linkage and the degree of branching, as well as the synergy between bacteria during fermentation, the relationship between substrate bacteria and fermentation products, the nature of the fermentations and the saccharolytic capacity of the bacteria (Voragen, 1998).

The production of oligosaccharides in food started to be investigated in Japan, between 1970-1975, and since then a number of these biopolymers have been identified (Table 1). The USA and Europe have recently become leaders in fructan, fructo-oligosaccharide (FOS) and inulin production. The reason is linked to their low cost production as well as the reproducibility of prebiotic effects in humans. Galacto-oligosaccharides (GOS) are also commercialized in these countries but not yet as widely used as fructans. (Rastall & Maitin, 2002).

The oligosaccharides have been widely used in foods, beverages and confectionery due to their properties as hygroscopicity, stabilization of active substances (involved in e.g. flavour

and colour), water activity, sweetness and bitterness. They can be obtained by three different ways: (i) extraction with hot water from roots (e.g. inulin) or seeds (e.g. soybean oligosaccharides), (ii) enzymatic synthesis from one or a mixture of disaccharides using osyl-transferases (e.g. fructooligosaccharides) or (iii) partial enzymatic hydrolysis of oligosaccharides (e.g. oligofructose) or polysaccharides (e.g. xylooligosaccharides) (Roberfroid & Slavin, 2000).

Compound	Molecular structure*	Raw material	Process
Cyclodextrins	(Glu) <sub>n</sub>	Starch	transglycosylation
Fructooligosaccharides	(Fru) <sub>n</sub> -Glu	Sucrose	transglycosylation
Galactooligosaccharides	(Gal) <sub>n</sub> -Glu	Lactose	transglycosylation
Gentiooligosaccharides	(Glu) <sub>n</sub>	Starch	Transglycosylation /hydrolysis
Glycosylsucrose	(Glu) <sub>n</sub> -Fru	Sucrose	transglycosylation
Isomaltooligosaccharides	(Glu) <sub>n</sub>	Starch	Transglycosylation /hydrolysis
Isomaltulose (palatinose)	(Glu-Fru) <sub>n</sub>	Sucrose	transglycosylation
Lactosucrose	Gal-Glu-Fru	Lactose/Sucrose	transglycosylation
Lactulose	Gal-Fru	Lactose	isomerization
Maltooligosaccharides	(Glu) <sub>n</sub>	Starch	hydrolysis
Raffinose	Gal-Glu-Fru	Sucrose	extraction
Stachyose	Gal-Gal-Glu-Fru	Sucrose	extraction
Soybean oligosaccharides	(Gal) <sub>n</sub> -Glu-Fru	Starch	extraction
Xylooligosaccharides	(Xyl) <sub>n</sub>	Xylan	hydrolysis

Table 1. Non-digestible oligosaccharides. \* Glu, glucose; Fru, fructose; Gal, galactose, Xyl, xylose

Polysaccharides are often the main source of bioactive oligosaccharides and therefore new sources of them are continuously investigated. In this context, LAB have become a promising target due to its GRAS/QPS status. Currently, bioactive commercialized oligosaccharides are extracted from plants but not yet from LAB. However, the high diversity of LAB and their EPS offer new possibilities for detection and production of bioactive oligosaccharides. To obtain oligosaccharides, the post-synthetic engineering strategies consist in enzymatic or chemical actions involving two types of enzymes, glycosyl-hydrolases (EC 3.2.1.y) and polysaccharide lyases (EC 4.2.2.y). A strategy to make the action of the enzyme specific is to grow the EPS-producing bacteria on plates with their own polysaccharide as a carbon source so that in order to survive, they themselves secrete

EPS degrading enzymes. After detection and purification, a specific enzyme for catalysis of the polysaccharide is obtained (Badel et al., 2011).

### 5.2.2 Immunomodulators

An immunomodulator is a substance which has an effect on the immune system. This system can be regulated in different ways by the use of immunosuppressors or immunostimulants to inhibit or to induce the immune responds. In particular, their use, included as additives in food, could be useful to combat infections, to prevent digestive tract cancers or to treat sicknesses due to immunodeficiency, such as inflammatory bowel diseases (Crohn's disease and ulcerative colitis). One strategy to modulate the immune system is the modulation of cytokine expression through the use of herbal medicines. The immunomodulators alter the activity of immune function through the dynamic regulation of informational molecules such as cytokines. (Spelman et al., 2006).

The mechanism involved in the immunomodulation can be explained by the interaction of the immunomodulators to their receptor in the membrane of an immune system cell. This interaction activates an internal cascade of phosphotranfer of proteins mediated by kinases and related to a specific pathway. As a consequence, a change of binding affinity of transcriptional regulators for their operators takes place, which results in activation or repression of gene expression (Figure 6).

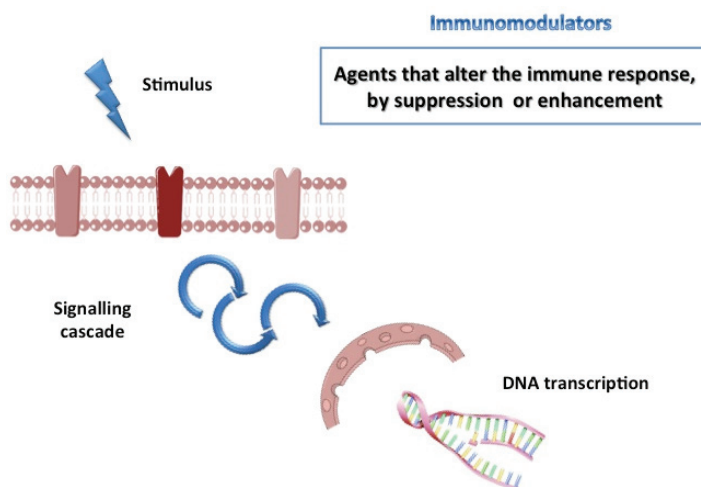


Fig. 6. stimulation and/or inhibition mediated by immunomodulators

Neither HoPS nor HePS produced by LAB have been used to elaborate functional food, nor directly tested as immunomodulators. However, most of the LAB used as probiotics (according to FAO/WHO, "live microorganisms which when administered in adequate amounts confer a health benefit on the host") for elaboration of functional food, have immunomodulatory properties (Foligne et al, 2007), and produce EPS. However, their ability to immunomodulate is strain specific and can not yet be directly connected to the EPS. In addition, current knowledge (see below) indicates that the nature and structure of

some of these biopolymers synthesized by LAB have the potential to be used as immunomodulating food additives.

$\beta$ -glucans are known as "biological response modifiers" (Wasser 2011), due to their ability to activate the immune system.  $\beta$ -glucans are integral cell wall components of a variety of fungi, plants and bacteria. In the early 40's Pillemer and Ecker (1941) described the effect of a crude yeast cell wall, called Zymosan, and described that this extract was able to activate the non-specific innate immunity. Only in the 80's Czop and Austin (1985) described the action of Zymosan that involved its binding to a  $\beta$ -1-3-glucan specific receptor, latter on identify and named Dectin-1 (Brown et al., 2001), found in the cell membrane of macrophages, which activates an internal cascade of events.

The immunomodulating function of the  $\beta$ -glucans is related to their structure; in fact different polysaccharides are able to interact with specific receptors, due to their 3D-structure. It have been recently demonstrated that  $\beta$ -glucans with a linear backbone containing 1-3 linkage (e. g. Zymosan) have the ability to activate several receptors: Dectin-1, complement receptor 3 (CR3), scavenger receptors class A and class B, and Toll-like receptors (TLRs) 2 and 6 (Chlubnova et al., 2011). The interaction with these receptors triggers a cascade of internal effects, including production of cytokines (TNF- $\alpha$ , IL-6 and IL-10).

Also branched 6-substituted (1-3)-  $\beta$ -glucans isolated from mushroom, *Candida albicans* and *Pneumocystis carinii*, show high affinity for Dectin-1 (Palma et al., 2006) and for TLR-2 and TLR-4 receptors in elicitation of immune response (Chlubnova et al. 2011). Mushroom, especially Basidiomycetes, are a source of  $\beta$ -glucans with a high biological activity. They mostly have a 1-3 linkage in the main chain, and sometime an additional 1-6 branch point. This branching point increase their antitumoral and immunomodulating effects (Barreto-Berger & Gorin, 1983). It is well established that the structural composition offers a higher capacity for carrying biological information, because they have a greater potential for structural variability, specially related to the triple-helical tertiary conformation (Yanaki et al., 1983). A good example of a preventive effect is given by a Japanese study on their popular edible and medicinal mushroom *Hypsizygus marmoreus* (Ikekawa, 2001). In this study mice were divided into two groups: untreated and treated with a diet containing 5% of the dried fruiting body of *H. marmoreus*. All mice were injected with a strong carcinogen, methyl-cholanthrene, and carcinogenesis was investigated. The results obtained allowed to the authors to conclude that the mechanism of prevention and inhibition of carcinogenesis was due to immunopotentialization (Ikekawa, 2001). Therefore, (1,3)  $\beta$ -glucan produced by LAB and their producing strains have potential as immunomodulators.

In this line, it has been shown that 2-substituted (1,3)  $\beta$ -glucan producer LAB belonging to the *Lactobacillus* and *Pediococcus* genera are able to immunomodulate macrophages *in vitro* (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010). Moreover, treatment of the macrophages with the purified biopolymer resulted in an increase of secretion of the anti-inflammatory IL-10 cytokine (Fernández de Palencia et al., unpublished results). In addition, four  $\beta$ -glucan-producing LAB strains have been tested for their survival under gastrointestinal stress (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010) , using an *in vitro* model that simulates conditions of human gut (Fernández de Palencia et al., 2008). Among them, *P. parvulus* 2.6 and *Lb. suebicus* CUPV221 showed significant

resistance to digestive tract gut conditions. Furthermore, the presence of the EPS conferred to the producing strains increased capability to adhere to Caco-2 human epithelial intestinal cells (Garai-Ibabe et al., 2010; Fernández de Palencia et al., 2009). Thus, the use of the 2-substituted (1,3)  $\beta$ -glucan as an additive, or produced *in situ*, in fermented food or in the gut has potential as an immunomodulator to alleviate inflammatory bowel diseases. In addition, human consumption of oat-based food prepared with *P. parvulus* 2.6 resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)- $\beta$ -D-glucans in oat (Martensson et al., 2005). Finally, the production of yogurt and various beverages with 2-substituted  $\beta$ -D-glucan-producer LAB indicate advantageous techno-functional properties of these strains (Elizaquível et al., 2011). Therefore, LAB producing this EPS have potential as probiotic strains useful for the manufacture of functional foods.

Dextran-70 has been also investigated as immunomodulators. Previous studies on dextran-70 justified its beneficial effect in the prevention of acute respiratory distress syndrome after trauma and sepsis as well as pancreatitis (Modig, 1988). Recently, it was reported that dextran-70 reduced the leukocyte-endothelium interaction. In a clinical trial forty patients who were undergoing coronary bypass surgery were divided into 2 groups of 20. In Group A a dextran-70 infusion was administered at a concentration of 7.5 ml kg<sup>-1</sup> before the surgery, and 12.5 ml kg<sup>-1</sup> after the cardiopulmonary bypass. Group B was the control and received a gelatin infusion at the same concentration. Several parameters were measured including determination of IL 8, IL 10 and troponin-I levels. The conclusion was that this  $\alpha$ -glucan was able to reduce the systemic inflammatory response and the release of the cardiac troponin-I after cardiac operation (Gombocz et al., 2007).

Another  $\alpha$ -glucan from the edible mushroom *Tricholoma matsutake* has been investigated, and reported to have excellent biological activities; exerting modulating effects on the immune competence of mice and rats. In this study, a sodium hydroxide extract of the mushroom was defatted followed by fractionation with a combination of ion exchange chromatography and gel filtration to identify the active component. A single-peak fraction (MPG-1) was obtained after reverse-phase chromatography. MPG-1 was a glycoprotein with molecular mass of 360 kDa, and contained about 90% glucose. NMR and methylation analysis revealed that the  $\alpha$ -1,4-linkage was the predominant glucan linkage with  $\alpha$ -1,6- and  $\alpha$ -1,2-branching in the minority. It was demonstrated that the mycelium preparation is effective in improving immunological functions in stressed individuals. In an *in vitro* model the compound formed a complex with the active form of TGF- $\beta$ 1. These results indicate that the mycelium contains a novel  $\alpha$ -glucan-protein complex with immunomodulatory effect (Hoshi et al., 2005).

Therefore, the high production of  $\alpha$ -glucans by LAB and the immunomodulatory properties of these bio-molecules as described above, predict that in the near future studies will be performed to evaluate the beneficial properties of these EPS, with the aim to use them as food additives.

Finally, the low yield of HePS produced by LAB and their complex biosynthetic pathway, suggests that in the short term they are not very good candidates as food additives, although it is expected that the immunomodulatory properties of the producing strains will be further investigated.

## 6. Conclusion

Currently it is clear that diversification of functional foods, which have been scientifically validated as having beneficial properties, will increase in the near future, and the EPS synthesized by LAB could have a place in the market as an ingredient of this type of food. To this end, EPS can be synthesized *in situ* by their producing strains or can be used in isolated form as a food additive. Their chances of reaching the marketplace will be improved by the discovery and utilization of new EPS-producing strains isolated from sources other than dairy products. The discovery and characterization of new EPS-producers isolated from food (e.g. processed meat products) and beverages (e.g. wine and cider) will increase the variety of EPS and the use of their producing strains for the elaboration of novel solid and liquid functional food. For example, *Lc. mesenteroides* Lb. *plantarum* and *Lb. sakei* strains have been isolated from Spanish sausages. They produce highly homogeneous,  $\alpha$ -glucan HoPS synthesised by a dextranucrase, and are able to immunomodulate macrophages (Nacher et al., unpublished results). Microorganisms that are native to the human gut and produce EPS (or could be engineered to produce EPS) would also be of great interest as their chances of survival in the gut environment would be much higher than other microbes. It has been shown that microorganisms that produce 2-substituted-(1,3)- $\beta$ -glucan are able to adhere to human gut epithelial cells. Such organisms would presumably be able to colonise the gut and compete effectively with pathogens, at the same time as producing a beneficial immunomodulation.

However, the use of EPS producing LAB strains will require a thorough scientific evaluation both *in vitro* and *in vivo*. It has to be stated that currently most of the general claims for components of functional food, (though this does not apply to oat  $\beta$ -glucans and their blood LDL-cholesterol lowering properties), have not been approved for use after evaluation by the European Food Safety Authority (EFSA, <http://www.efsa.europa.eu>). The main reason for rejection of probiotic bacteria has been the lack of enough characterization of the bacteria (determination of the nucleotide sequence of their entire genome is advisable), and/or insufficient scientific evidence to correlate the use of the bacteria with health benefits (*Lb. delbrueckii* subsp. *bulgaricus* AY/CSL and *S. thermophilus* 9Y/CSL and beneficial modulation of the gut microbiota). Therefore, each particular strain has to be subjected to evaluation, although its EPS has been already experimentally validated. The evaluation should be performed, first *in vitro*, then in animal models and finally in human trials. In addition, due to the rules of the EFSA the use of genetically modified organisms (GMO) is restricted (although they are not totally forbidden). Moreover, the opinion of consumers in Europe and USA regarding the use of GMO in food is not favourable. Consequently, well-characterized (preferably GRAS) EPS-producers from natural ecological environments are the best candidates for use in functional food.

However, if the EPS are going to be used as food additives, after purification, then there is no restriction concerning the use of a GMO producing strain. The use of GMO able to produce high levels of EPS or newly designed biopolymers is still very limited, and the production levels of most EPS are not very high. Therefore, provided that enzymes, and hence the genes, involved in their biosynthetic pathways are known, the future improvement of EPS production, will be by DNA recombinant technology and metabolic engineering to generate GMO EPS-producing LAB, that will be used for production of the biopolymers in large-scale fermenters. Moreover, genetic engineering could be used to alter

substrate specificity of the EPS biosynthetic enzymes to generate new polysaccharides and oligosaccharides with improved prebiotic properties. In the case of the glycosyltransferases, which synthesize HoPS this strategy could result in the synthesis of not only new HoPS, but also new HePS. Finally, the requirement of only one protein for the synthesis opens the window for production of new biopolymers by immobilized enzymes.

Overall, there should be a rapid expansion in the development of novel LAB probiotic organisms and their prebiotic EPS products. However, their ultimate success in the market place will require a rigorous scientific evaluation.

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## 8. References

- Adeyeye, A., Jansson, P.E. & Lindberg, B. (1988). Structural studies of the capsular polysaccharide from *Streptococcus pneumoniae* type 37. *Carbohydrate Research*, Vol.180, No.2, (September 1988), pp. 295-299
- Angelov, A., Gotcheva, V., Hristozova, T. & Gargova, S. (2005). Application of pure mixed probiotic lactic acid bacteria and yeast cultures for oat fermentation. *Journal of the Science of Food and Agriculture*, Vol.85, No.12, (September 2005), pp. 2134-2141
- Aslan, Y. & Tanriseven, A. (2007). Immobilization of *Penicillium lilacinum* dextranase to produce isomaltooligosaccharides from dextran. *Biochemical Engineering Journal*, Vol.34, No.1, (April 2007), pp. 8-12
- Badel, S., Bernardi, T. & Michaud, P. (2011). New perspectives for Lactobacilli exopolysaccharides. *Biotechnology Advances*, Vol.29, No.1, (January-February 2011), pp. 54-66
- Barreto-Bergter, E. & Gorin, P.A. J. (1983). Structural chemistry of polysaccharides from fungi and lichens In: *Advances in Carbohydrate Chemistry and Biochemistry*, R.S. Tipson and H. Derek, pp.(67-103), Academic Press, ISBN: 0065-2318
- Boels, I.C., Kleerebezem, M. & de Vos, W.M. (2002). Engineering of carbon distribution between glycolysis and sugar nucleotide biosynthesis in *Lactococcus lactis*. *Applied Environmental Microbiology*, Vol.69, No.2 (February 2002), pp. 1129-1135
- Bourgoin, F., Pluvinet, A., Gintz, B., Decaris, B. & Guédon, G. (1999). Are horizontal transfers involved in the evolution of the *Streptococcus thermophilus* exopolysaccharide synthesis loci?. *Gene*, Vol.233, No.1-2, (June 1999), pp. 151-161
- Brock, T.D. (2008). *Biology of Microorganisms*. 12th ed. M. Madigan, J. Martinko, J. Parker. Pearson/Prentice-Hall. ISBN 0321536150
- Brown, G.D. and Gordon, S. (2001). A new receptor for  $\beta$ -glucans. *Nature*. Vol.413, (September 2001), pp. 36-37
- Capek, P., Hlavonová, E., Matulová, M., Mislovicová, D. Ruzicka, J., Koutný, M. & Keprdová, L. (2011).



- 1 Isolation and characterization of an extracellular glucan produced by *Leuconostoc garlicum*  
2 PR. *Carbohydrate Polymers*, Vol.83, No.1, (January 2011), pp. 88-93
- 3 Charnock, S.J., Henrissat B. & Davies, G.J. (2001). Three-dimensional structures of UDP-  
4 sugar glycosyltransferases illuminate the biosynthesis of plant polysaccharides.  
5 *Plant Physiology*, Vol.125, No.2, (February 2003), pp. 527-531
- 6 Chlubnova, I., Sylla, B., Nugier-Chauvin, C., Daniellou, R., Legentil, L., Kralova, & B.,  
7 Ferrieres, V. (2011). Natural glycans and glycoconjugates as immunomodulating  
8 agents. *Natural Product Reports*, Vol.28, No. 5, (March 2011), pp. 937-952
- 9 Ciucanu, I. & Kerek, F. (1984). Rapid and simultaneous methylation of fatty and hydroxy  
10 fatty acids for gas-liquid chromatographic analysis. *Journal of Chromatography A*,  
11 Vol. 284, (1984), pp.179-185
- 12 Coutinho, P.M., Deleury E., Davies G.J. & Henrissat B. (2003). An Evolving Hierarchical  
13 Family Classification for Glycosyltransferases. *Journal of Molecular.Biology*, Vol.328,  
14 No.2, (April 2003), pp. 307-317
- 15 Czop, J. & Austen K. (1985). A  $\beta$ -glucan inhibitable receptor on human monocytes: its  
16 identity with the phagocytic receptor for particulate activators of the alternative  
17 complement pathway. *The Journal of Immunology*, Vol.134, No. 4, (April 1985), pp.  
18 2588-2593
- 19 Dabour, N. & LaPointe, G. (2005). Identification and molecular characterization of the  
20 chromosomal exopolysaccharide biosynthesis gene cluster from *Lactococcus lactis*  
21 *subsp. cremoris* SMQ-461. *Applied .and Environmental Microbiology*, Vol.71, No.11,  
22 (November 2005), pp. 7414-7425
- 23 de Vuyst, L. & Degeest, B. (1999). Heteropolysaccharides from lactic acid bacteria. *FEMS*  
24 *Microbiology Reviews*, Vol.23, No.2, (April 1999), pp. 153-17
- 25 de Vuyst, L., De Vin, F., Vaningelmem, F. & Degeest B. (2001). Recent developments in the  
26 biosynthesis and applications of heteropolysaccharides from lactic acid bacteria.  
27 *International Dairy Journal*, Vol.11, No.9, (October 2001), pp. 687-707
- 28 Dols-Lafargue, M., Lee, H.Y., Le Marrec, C., Heyraud, A., Chambat, G. & Lonvaud-Funel, A.  
29 (2008). Carcterization of *gtf*, a glucosyltransferase gene in the genome of  
30 *Pediococcus parvulus* and *Oenococcus oeni*, two bacterial species commonly found in  
31 wine. *Applied and Environmental Microbiology*, Vol.74, No.13, (July 2008), pp. 4079-  
32 4090
- 33 DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., & Smith, F. (1956). Colorimetric  
34 Method for Determination of Sugars and Related Substances. *Analytical Chemistry*,  
35 Vol.28, No. 3, (March 1956) , pp. 350-356
- 36 Duus, J.Ø., Gotfredsen, C.H. & Bock, H. (2000). Carbohydrate structural determination by  
37 NMR spectroscopy: modern methods and limitations. *Chemical Reviews*, Vol.100,  
38 No.12, (November 2000), pp. 4589-4614
- 39 Dueñas, M., Munduate, A., Perea, A., & Irastorza, A. (2003). Exopolysaccharide production  
40 by *Pediococcus damnosus* 2.6 in a semidefined medium under different growth  
41 conditions. *International Journal of Food Microbiology*, Vol. 87, No. 1-2, (October 2003),  
42 pp. 113-120
- 43 Dueñas-Chasco, M.T., Rodríguez-Carvajal, M.A., Mateo, P.T., Franco-Rodríguez, G.,  
44 Espartero, J., Irastorza-Iribas, A. & Gil-Serrano, A.M. (1997). Structural analysis of  
45 the exopolysaccharide produced by *Pediococcus damnosus* 2.6. *Carbohydrate Research*,  
46 Vol.303, No.4, (October 1997), pp. 453-458

- Dueñas-Chasco, M.T., Rodríguez-Carvajal, M.A., Tejero-Mateo, P., Espartero, J.L., Irastorza-Iribas, A. & Gil-Serrano, A.M., (1998). Structural analysis of the exopolysaccharides produced by *Lactobacillus* spp. G-77. *Carbohydrate Research*, Vol.307, No.1-2, (February 1998), pp. 125-133
- Duboc, P. & Mollet, B. (2001). Applications of exopolysaccharides in the dairy industry. *International Dairy Journal*. Vol.11, No.9, (October 2001), pp. 759-768.
- Elizaquível, P., Sánchez, G., Salvador, A.I., Fiszman, S., Dueñas, M.T., López, P. Fernández de Palencia, P. & Aznar, R. (2011). Evaluation of yogurt and various beverages as carriers of lactic acid bacteria producing 2-branched (1,3)- $\beta$ -D-glucan. *Journal of Dairy Science*, Vol.94, No. 7 (July 2011), No. pp. 3271-3278
- Erhardt, F.A., Kügler, J., Chakravarthula, R.R. & Jördening, H.J. (2008). Co-Immobilization of dextranucrase and dextranase for the facilitated synthesis of isomaltoligosaccharides: preparation, characterization and modeling. *Biotechnology and Bioengineering*, Vol.100, No.4, (July 2008), pp. 673-683
- Fernández de Palencia, P., López, P., Corbí, A., Peláez, C., & Requena, T. (2008). Probiotic strains: survival under simulated gastrointestinal conditions, in vitro adhesion to Caco-2 cells and effect on cytokine secretion. *European Food Research and Technology*, Vol. 227, No.5, (March 2008), pp. 1475-1484.
- Foligne, B., Nutton, S., Grangette, C., Dennin, V., Goudercourt, D., Poirer, S., Dewulf, J., Brassart, D., Mercenier, A. & Pot, B. (2007). Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. *World Journal of Gastroenterology*, Vol.14, No.2, (January 2007), pp. 236-243
- Folkenberg, D.M., Dejmeck, P., Skriver, A., Guldager, H.S. & Ipsen, R. (2006). Sensory and rheological screening of exopolysaccharide producing strains of bacterial yoghurt cultures *International Dairy Journal*, Vol.16, No.2, (February 2006), pp. 111-118
- Garai-Ibabe, G., Areizaga, J., Aznar, R., Elizaquível, P. Prieto, A., Irastorza, A. & Dueñas, M.T. (2010). Screening and selection of 2-branched (1,3)- $\beta$ -D-glucan producing lactic acid bacteria and exopolysaccharide characterization. *Journal of Agriculture Food Chemistry*, Vol. 58, No. 10, (December 2010), pp. 6149-6156
- Garai-Ibabe, G., Werning, M.L., López, P., Corbí, A.L. & Fernández de Palencia, P. (2010). Naturally occurring 2-substituted (1,3)- $\beta$ -D-glucan producing *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the food industry. *Bioresource Technology*, Vol.101, No.23, (December 2010), pp. 9254-9263
- García, E., Llull, D., Muñoz, R., Mollerach M. & López R. (2000) Current trends in capsular polysaccharide biosynthesis of *Streptococcus pneumoniae*. *Research in Microbiology*, Vol.151, No.6, (July 2000), pp. 429-435
- Garinot-Schneider, C., Lellouch, A.C. & Geremia, R.A. (2000) Identification of essential amino acid residues in the *Sinorhizobium meliloti* glucosyltransferase ExoM. *Journal of Biological Chemistry*, Vol.183, No.1, (October 2000), pp. 31407-31413
- Gänzle, M., & Schwab, C. (2009). Ecology of exopolysaccharide formation by lactic acid bacteria: sucrose utilisation, stress tolerance, and biofilm formation, In: *Bacterial Polysaccharides: Current Innovations and Future Trends*, Matthias Ullrich, pp. (263-278) Caister Academic Press, ISBN 978-1-904455-45-5, Norfolk, UK.
- Gibson, G.R. & Roberfroid, M. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition*, Vol.125, No.6, (June 1995), pp. 1401-1412

- Gibson, G.R., Probert, H.M., Van Loo, J., Rastall, R.A. & Roberfroid, M. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews*, Vol.17, No. 2, (December 2004), pp. 259-275
- Glucksmann, M.A., Reuber, T.L. & Walker, G.C. (1993). Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. *Journal of Bacteriology*, Vol.175, No.21, (November 1993), pp. 7045-7055
- Gombocz, K., Beledi, A., Alotti, N., Kecskes, G., Gabor, V., Bogar, L., Koszegi, T., & Garai, J. (2007). Influence of dextran-70 on systemic inflammatory response and myocardial ischaemia-reperfusion following cardiac operations. *Critical Care*, Vol. 11, No. 4, (August 2007), pp. R87
- Goulas, A. K., Cooper, J.M, Grandison, A.S., & Rastall, R.A. (2004). Synthesis of isomaltooligosaccharides and oligodextrans in a recycle membrane bioreactor by the combined use of dextranase and dextranase. *Biotechnology and Bioengineering*, Vol. 88, No. 6, (December 2004), pp. 778-787
- Heldermon, C.; DeAngelis, P.L. & Weigel, P.H. (2001). Topological organization of the hyaluronan synthase from *Streptococcus pyogenes*. *Journal of Biological Chemistry*, Vol.276, No.3, (January 2001), pp. 2037-2046
- Hoshi, H., Yagi, Y., Iijima, H., Matsunaga, K., Ishihara, Y., & Yasuhara, T. (2005). Isolation and characterization of a novel immunomodulatory alpha-glucan-protein complex from the mycelium of *Tricholoma matsutake* in basidiomycetes. *Journal of Agricultural and Food Chemistry*, Vol. 53, No. 23, (November 2005), pp. 8948-8956
- Ibarburu, I., Soria-Diaz, M.E.; Rodriguez-Carvajal, M.A., Velasco, S.E., Tejero-Mateo, P., Gil-Serrano, A. M., Irastorza, A. & Dueñas, M.T. (2007). Growth and exopolysaccharide (EPS) production by *Oenococcus oeni* I4 and structural characterization of their EPSs. *Journal of Applied Microbiology*, Vol.103, No.2, (August 2007), pp. 477-486
- Ikeda, S. & Shisido, Y. 2005. Atomic force studies on heat-induced gelation of curdlan. *Journal of Agricultural Chemistry*, Vol.53, No.3 (January 2005), pp. 786-791
- Ikekawa, T. (2001). Beneficial Effects of Edible and Medicinal Mushrooms. *Health Care*, Vol.3, No.4, (December 2001), pp. 8
- Jolly, L. & Stinglele, F. (2001). Molecular organization and functionality of exopolysaccharide gene clusters in Lactic acid bacteria. *International Dairy Journal*, Vol.11, No.9, (October 2001), pp. 733-745
- Karnezis, T.; Epa, V. C., Stone, B. A. & Stanisich, V. A. (2003). Topological characterization of an inner membrane (1->3)-β-D-glucan (curdlan) synthase from *Agrobacterium* sp. strain ATCC31749. *Glycobiology*, Vol.13, No.10, (October 2003), pp. 693-706
- Keenleyside, W.J., Clarke, A.J. & Whitfield, C. (2001). Identification of Residues Involved in Catalytic Activity of the Inverting Glycosyl Transferase WbbE from *Salmonella enterica* serovar borreze. *Journal of Bacteriology*, Vol.183, No.1, (January 2001), pp. 77-85
- Kimmel, S.A., Roberts, R.F. & Ziegler, G.R. (1998). Optimization of exopolysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* RR grown in a semidefined medium. *Appl Environ Microbiol* Vol. 64, No. 2, (February 1998), pp. 659-664
- Kleerebezem, M., van Kranenburg, R., Tuinier, R., Boels, I.C., Zoon, P., Looijesteijn, E., Hugenholtz, J. & de Vos, W.M. (1999). Exopolysaccharides produced by *Lactococcus*

- 1 *lactis*: from genetic engineering to improved rheological properties? *Antonie van*  
2 *Leeuwenhoek*, Vol.76, No.1-4, (July-November 1999), pp. 357-365
- 3 Korakli, M. & Vogel, R.F. (2006). Structure/function relationship of homopolysaccharide  
4 producing glycansucrases and therapeutic potential of their synthesized glycans.  
5 *Applied Microbiology and Biotechnology*, Vol.71, No.6, (April 1986), pp. 790-803
- 6 Kuipers, O. P., de Ruyter, P. G. G. A., Kleerebezem, M. & de Vos, W. M. (1998). Quorum  
7 sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*,  
8 Vol.64, No.1, (September 1998), pp. 15-21
- 9 Laine, R. A., Esselman, W.J., & Sweeley, C.C. (1972). Gas-liquid chromatography of  
10 carbohydrates. In: *Methods in Enzymology*. Victor, Ginsburg, pp. 159-167, Academic  
11 Press, ISBN: 0076-6879
- 12 Lairson, L. L., Henrissat, B., Davies, G.J. & Withers, S.G. (2008). Glycosyltransferases:  
13 structures, functions, and mechanisms. *Annual Review of Biochemistry*, Vol.77, (July  
14 2008), pp. 521-555
- 15 Lamothe, G. T., Jolly, L., Mollet, B. & Stingle, F. (2002). Genetic and biochemical  
16 characterization of exopolysaccharide biosynthesis by *Lactobacillus delbruekii* subsp.  
17 *Bulgaricus*. *Archives of Microbiology*, Vol. 178, No.3, (September 2002), pp. 218-228.
- 18 Laws, A, Gu, Y. & Marshall, V. (2001) Biosynthesis, characterisation, and design of bacterial  
19 exopolysaccharides from lactic acid bacteria. *Biotechnology Advances*, Vol.19, No.8,  
20 (December 2001), pp. 597-62
- 21 Leal, D., B. Matsushiro, B., Rossi, M., & Caruso, F. (2008). FT-IR spectra of alginic acid block  
22 fractions in three species of brown seaweeds. *Carbohydrate Research*, Vol.343, No.2,  
23 (2008), pp. 308-316
- 24 Lin, W.S.; Cunneen, T. & Lee, C.Y. (1994). Sequence analysis and molecular characterization  
25 of genes required for the biosynthesis of type 1 capsular polysaccharide in  
26 *Staphylococcus aureus*. *Journal of Bacteriology*, Vol.176, No.22, (November 1994), pp.  
27 7005-7016
- 28 Liu, J.R.; Wang, S.Y., Lin, Y.Y. & Lin, C.W. (2002). Antitumor activity of milk-kefir and  
29 soy milk-kefir in tumor-bearing mice. *Nutrition and Cancer*, Vol.44, No.2, (Nov 2009),  
30 pp. 182-187
- 31 Llaubères, R. M., Richard B., Lonvaud, A., Dubourdieu, D. & Fournet, B. (1990). Structure of  
32 an exocellular  $\beta$ -D-glucan from *Pediococcus* sp, a wine lactic bacteria. *Carbohydrate*  
33 *Research*, Vol.203, No.1, (August 1990), pp. 103-107
- 34 Llull, D.; Garcia, E. & Lopez, R. (2001). Tts, a processive  $\beta$ -glucosyltransferase of  
35 *Streptococcus pneumoniae*, directs the synthesis of the branched type 37 capsular  
36 polysaccharide in pneumococcus and other gram-positive species. *Journal of*  
37 *Biological Chemistry*, Vol.276, No.24, (June 2001), pp. 21053-21061
- 38 Macedo, M.G., Lacroix, C. & Champagne, C.P. (2002). Combined effects of temperature and  
39 medium composition on exopolysaccharide production by *Lactobacillus rhamnosus*  
40 rw-9595m in a whey permeate based medium. *Biotechnological Progress*, Vol.18,  
41 No.2, (Mar-Apr 2002), pp.167-173
- 42 Modig, J. (1988). Comparison of effects of dextran-70 and ringer's acetate on pulmonary  
43 function, hemodynamics, and survival in experimental septic shock. *Critical Care*  
44 *Medicine*, Vol.16, No.3, (March 1988), pp. 266-271
- 45 Mateo, C., Palomo, M., Fernandez-Lorente, G., Guisan, J.M. & Fernandez-Lafuente, R.  
46 (2007). Improvement of enzyme activity, stability and selectivity via immobilization

- techniques. *Enzyme and Microbial Technology*, Vol.40, No.6, (May 2007), pp. 1451-1463
- Marshall, V.M. & Rawson, H.L. (1999). Effects of exopolysaccharide-producing strains of thermophilic lactic acid bacteria on the texture of stirred yoghurt. *International Journal of Food Science and Technology*, Vol.34, No. 2, (April 1999), pp. 137-143
- Martensson, O., Björklund, M., Lambo, M. A., Dueñas-Chasco, M. T., Irastorza, A., Holst, O., Norin, E., Walling, G., Öste, R. & Önning, G. (2005). Fermented ropy, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. *Nutrition Research*, Vol.25, No.5, (May 2005), pp. 429-442
- McIntosh, M., Stone, B.A. & Stanisich, V.A. (2005). Curdlan and other bacterial (1,3)-  $\beta$ -D-glucans. *Applied Microbiology and Biotechnology*, Vol.68, No.2, (August 2005), pp. 163-173
- Monsan, P., Bozonnet, S., Albenne, C., Joucla, G., Willemot R.-M. & Remaud-Simeon, M. (2001). Homopolysaccharides from lactic acid bacteria. *International Dairy Journal*, Vol.11, No.9, (October 2001), pp. 675-685
- Monterisino, S., Prieto, A., Muñoz, R. & de las Rivas, B. (2008). Evaluation of exopolysaccharide production by *Leuconostoc mesenteroides* strains isolated from wine. *Journal of Food Science*, Vol.73, No.4, (May 2008), pp. 196-199
- Mozzi, F., Vaningelgem, F., Hebert, E. M., Van der Meulen, R., Foulquie Moreno, M. R., Font de Valdez, G. & De Vuyst, L. (2006). Diversity of heteropolysaccharide-producing lactic acid bacterium strains and their biopolymers. *Applied and Environmental Microbiology*, Vol.72, No.6, (June 2006), pp. 4431-4435
- Mussatto, S.I. & Mancilha, I.M. (2007). Non-digestible oligosaccharides: A review. *Carbohydrate Polymers*, Vol.68, No. 3, (April 2007), pp. 587-597
- Nordmark, E. L., Yang Z., Huttunen, E. & Widmalm G., (2005). Structural studies of the exopolysaccharide produced by *Propionibacterium freudenreichii* ssp. *shermanii* JS. *Biomacromolecules*, Vol.6, No.1, (November 2004), pp. 521-523
- Ölcer, Z. & Tanriseven, A. (2010). Co-immobilization of dextranucrase and dextranase in alginate. *Process in Biochemistry*, Vol.45, No. 10, (October 2010), pp. 1645-1651
- Palma, A.S., Feizi, T., Zhang, Y., Stoll, M.S., Lawson, A.M., Díaz-Rodríguez, E., Campanero-Rhodes, M.A., Costa, J., Gordon, S., Brown, G.D., &Chai, W. (2006). Ligands for the  $\beta$ -glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. *Journal of Biological Chemistry*, Vol.281, No.9, (March 2006), pp. 5771-5779
- Péant, B., LaPointe, G., Gilbert, C., Atlan, D., Ward, P. & Roy, D. (2005). Comparative analysis of the exopolysaccharide biosynthesis gene clusters from four strains of *Lactobacillus rhamnosus*. *Microbiology*, Vol.151, No.6, (June 2005), pp. 1839-1851
- Pillemer, L. and Ecker, E. E. (1941). Anticomplementary factor in fresh yeast. *Journal of Biological Chemistry*, Vol.137, No.1, (January 1941), pp. 139-142
- Rastall, R.A & Maitin, V. (2002). Prebiotics and synbiotics: towards the next the generation. *Current Opinion in Biotechnology*, Vol.13, No.5, (October 2002), pp. 490-496
- Ricciardi, A. & Clement, F. (2000). Exopolysaccharides from lactic acid bacteria: structure, production and technological applications. In: *Italian Journal of Food Science*, Vol.12, No.1, (June 2000), pp. 23-45, ISSN 1120-1770

- Roberfroid, M. & Slavin, J. (2000). Nondigestible oligosaccharides. *Critical Reviews in Food Science and Nutrition*, Vol.40, No.6, (November 2000), pp. 461-480
- Ruas-Madiedo, P. & de los Reyes-Gavilan, C.G. (2005). Invited review: methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science*, Vol.88, No.3 (March 2005), pp. 843-856
- Ruas-Madiedo, P., Abraham, A., Mozzi, F. & de los Reyes-Gavilán, C.G. (2008). Functionality of exopolysaccharides produced by lactic acid bacteria, In: *Molecular aspects of lactic acid bacteria for traditional and new applications*. B. Mayo, P. López, and G. Pérez-Martín (Ed.), 137-166, Research Signpost, ISSN 978-81-308-0250-3, Kerala, India
- Ruas-Madiedo, P., Gueimonde, M., Arigoni, F., de los Reyes-Gavilan, C.G. & Margolles, A. (2009) Bile Affects the Synthesis of Exopolysaccharides by *Bifidobacterium animalis*. *Applied and Environmental Microbiology*, Vol.75, No.4, (February 2009), pp. 1204-1207
- Ruas-Madiedo, P., Salazar, N. & de los Reyes-Gavilan, C.G. (2009) Biosynthesis and chemical composition of exopolysaccharides produced by lactic acid bacteria, In: *Bacterial Polysaccharides: Current Innovations and Future Trends*, Matthias Ullrich (Ed.), pp. (279-310), Caister Academic Press, ISBN 978-1-904455-45-5, Norfolk, UK
- Sarwat, F., Ul Qader, S.A., Aman, A. & Ahmed, N. (2008). Production & characterization of a unique dextran from an indigenous *Leuconostoc mesenteroides* cmg713. *International Journal of Biological Sciences*, Vol.4, No.6, (July 2008), pp. 379-386.
- Silver, R.P., Prior, K., Nsahlai, C. & Wright, L.F. (2001). ABC transporters and the export of capsular polysaccharides from Gram-negative bacteria. *Research in Microbiology*, Vol.152, No.3-4, (April-May 2001), pp. 357-364
- Spelman, K., Burns, J., Nichols, D., Winters, N., Ottersberg, S., & Tenborg, M. (2006). Modulation of cytokine expression by traditional medicines: A review of herbal immunomodulators. *Alternative Medicine Review*, Vol.11, No.2, (June 2006) pp.128-150.
- Sletmoen, M. & Stokke, B. T. (2008). Higher order structure of (1,3)- $\beta$ -D-glucans and its influence on their biological activities and complexation abilities. *Biopolymers*, Vol.89, No.4, (January 2008), pp. 310-321
- Stack, H.M., Kearney, N., Stanton, C., Fitzgerald, G.F. & Ross, R.P. (2010). Association of  $\beta$ -glucan endogenous production with increased stress tolerance of intestinal Lactobacilli. *Applied and Environmental Microbiology*, Vol.76, No.2, (January 2010), pp. 500-507
- Stinge, F., Newell, J.W. & Neeser, J.R. (1999). Unraveling the function of glycosyltransferases in *Streptococcus thermophiles* Sfi6. *Journal of Bacteriology*, Vol.181, No.20, (October 1999), pp. 6354-6360
- Tanriseven, A. & Dogan S., (2002). Production of isomalto-oligosaccharides using dextransucrase immobilized in alginate fibres. *Process Biochemistry*, Vol. 37, (October 2001), pp. 1111-1115
- Tarbouriech, N., Charnock, S.J. & Davies, G.J. (2001). Three-dimensional structures of the Mn and Mg dTDP complexes of the family GT-2 glycosyltransferase SpsA: a comparison with related NDP-sugar glycosyltransferases. *Journal of Molecular Biology*, Vol.314, No.4, (December 2001), pp. 655-661
- Tieking, M., Korakli, M., Ehrmann, M.A., Gänzle, M.G. & Vogel, R.F. (2003). In situ production of exopolysaccharides during sourdough fermentation by cereal and

- intestinal isolates of lactic acid bacteria. *Applied and Environmental Microbiology*, Vol.69, No.2, (February 2003), pp. 945-952
- Tieking, M.; Ehrmann, M.A., Vogel, R.F. & Gänzle, M.G. (2005). Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392. *Applied Microbiology and Biotechnology*, Vol.66, No.6, (March 2005), pp. 655-663
- Tlapak-Simmons, V. L.; Baggenstoss, B. A.; Clyne, T. & Weigel, P. H. (1999). Purification and lipid dependence of the recombinant hyaluronan synthases from *Streptococcus pyogenes* and *Streptococcus equisimilis*. *Journal of Biological Chemistry*, Vol.274, No.7, (February 1999), pp. 4239-4245
- Tlapak-Simmons, V. L., Kempner, E. S., Baggenstoss, B.A. & Weigel, P.H. (1998). The active streptococcal hyaluronan synthases (HASs) Contain a Single HAS Monomer and multiple cardiolipin molecules. *Journal of Biological Chemistry*, Vol.273, No.40, (October 1998), pp. 26100-26109
- Van der Meulen, R., Grosu-Tudor, S., Mozzi, F., Vaningelgem, F., Zamfir, M., Font de Valdez, G. & De Vuyst, L. (2007). Screening of lactic acid bacteria isolates from dairy and cereal products for exopolysaccharide production and genes involved. *International Journal of Food Microbiology*, Vol.118, No.3, (July 2007), pp. 250-258
- van Hijum, S.A.F.T., Kralj, S., Ozimek, L.K., Dijkhuizen, L. & van Geel-Schutten, I.G.H. (2006). Structure-function Relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiology and Molecular Biology Reviews*, Vol.70, No.1, (March 2006), pp. 157-176
- van Hijum, S.A.F.T., Szalowska, E., van der Maarel, M.J.E.C. & Dijkhuizen, L. (2004). Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. *Microbiology*, Vol.150, No.3, (March 2004), pp. 621-630
- Velasco, S. E., Areizaga, J., Irastorza, A., Dueñas, M.T., Santamaria, A. & Muñoz, M.E. (2009). Chemical and rheological properties of the  $\beta$ -glucan produced by *Pediococcus parvulus* 2.6. *Journal of Agricultural and Food Chemistry*, Vol.57, No.5, (March 2009), pp. 1827-1834
- Vijayendra, S.V. & R.S. Babu R.S. (2008). "Optimization of a new heteropolysaccharide production by a native isolate of *Leuconostoc* sp. Cfr-2181. *Letters in Applied Microbiology*, Vol.46, No.6, (Jun 2008) pp.643-648
- Voragen, A.G.J. (1998). Technological aspects of functional food-related carbohydrates. *Trends in Food Science and Technology*, Vol.9, No. 8-9, (August 1998) pp. 328-335
- Wasser, S.P. (2011). Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. *Applied Microbiology and Biotechnology*, Vol.89, No.5, (Mar 2011) pp.1323-1332
- Walling, E., Gindreau, M. & Lonvaud-Funel, A. (2001). La biosynthèse d'exopolysaccharide par des souches de *Pediococcus damnosus* isolées du vin: mise au point d'outils moléculaires de détection. *Lait*, Vol.81, No.1-2, (January-April 2001), pp. 289-300
- Werning, M.L., Ibarburu, I., Dueñas, M.T., Irastorza, A., Navas, J. & López, P. (2006). *Pediococcus parvulus* gtf gene encoding the GTF glycosyltransferase and its application for Specific PCR detection of  $\beta$ -D-glucan-producing bacteria in foods and beverages. *Journal of Food Protection*, Vol.69, No.1, (January 2006), pp. 161-169
- Werning, M.L., Corrales, M.A., Prieto, A., Fernández de Palencia, P., Navas, J., & López, P. (2008). Heterologous expression of a position 2-substituted (1 $\rightarrow$ 3)-  $\beta$ -glucan in

- 1 *Lactococcus lactis*. *Applied Environmental Microbiology*, Vol.74, No.16, (Aug 2008)  
2 pp.5259-5262
- 3 Wood, B.J.B. (1997). *Microbiology of fermented food*, Blackie Academic & Professional, ISBN 0-  
4 7514-0216-8, London, United Kingdom
- 5 Wu, J., Zhang, Y., Wang, L., Xie, B., Wang, H. & Deng, S. (2006). Visualization of single and  
6 aggregated hulless oat (*Avena nuda* L.) (1→3),(1→4)-β-d-glucan molecules by  
7 atomic force microscopy and confocal scanning laser microscopy. *Journal of*  
8 *Agriculture Food Chemistry*, Vol.54, No.3, (January 2005), pp. 925-934
- 9 Yanaki, T., Ito, W., Tabata, K., Kojima, T., Norisuye, T., Takano, N. & Fujita, H. (1983).  
10 Correlation between the antitumor activity of a polysaccharide schizophyllan and  
11 its triple-helical conformation in dilute aqueous solution. *Biophysical Chemistry*,  
12 Vol.17, No.4, (June 1983), pp. 337-342